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STATE-DEPENDENT CHANGES OF ENDOGENOUS OPIOID ACTIVITY IN MAMMALIAN HIBERNATION

by

YAN CUI ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Biological Sciences

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Dr. Christina I. Benishin

Dr. John P. Chang

Dr. Glen Baker

for Dr. Alexander L. Beckman

September 26, 1995

This thesis is dedicated to

my husband

and

my parents.

ABSTRACT

The possible functional roles of various endogenous opioids in regulating hibernation were investigated by monitoring changes of endogenous opioid activity, including alterations in sensitivity of opioid receptor to exogenous opioids in physiological and pharmacological responses, receptor binding, and the content and apparent turnover rates of endogenous opioids in the limbic system throughout an annual hibernation cycle.

In vitro brain slice perifusion studies demonstrated that both μ and δ agonists inhibited, while the κ agonist enhanced K^+-stimulated 5-HT release from the hippocampus of non-hibernating ground squirrels. The inhibition by a δ agonist and stimulation by a κ agonist on 5-HT release was attenuated during hibernation but the inhibitory modulation of a μ agonist remained unaltered. In the septum of non-hibernating animals, *in vivo* microdialysis studies indicated only the δ agonist stimulated 5-HT apparent turnover but the stimulation was also diminished during hibernation. Together, the minimization of responsiveness of δ and κ receptors on K^+-stimulated 5-HT release and δ receptors on 5-HT apparent turnover indicates a decrease in opioid receptor sensitivity during hibernation. This was further supported by a decrease in thermoregulatory responsiveness to intra-hippocampal application of δ agonist in the hibernation season.

Quantitative receptor autoradiography provided direct evidence of a significant reduction in the binding of μ and δ receptors in the septo-hippocampal area of the hibernating animals. A decrease in κ receptor binding was also observed in the claustrum and CA3 of the hippocampus during hibernation. Associated with the decreases in opioid receptor responsiveness, endogenous opioid content of the corresponding CNS regions increased during hibernation. Microdialysis further

showed that the elevation of endogenous opioid contents were due to a rise in extracellular opioid concentration in the hibernating state and a significant increase in apparent turnover of dynorphin A and β-endorphin was also observed during hibernation after correcting for the Q_{10} effect.

In conclusion, the present study indicates that the reduction of opioid receptor efficacy during hibernation is due to an increased endogenous opioid activity. The changes in opioid concentration and opioid receptor sensitivity and binding are state-dependent, type-specific, and site-specific. This implies that different opioids may play different roles in entry, maintenance, and arousal from hibernation and they may elicit their effects by modulating the activity of other neurotransmitters, such as 5-HT.

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CHAPTER ONE

Introduction

Under seasonal cold and food shortage, many small mammals and birds utilize torpor or hibernation to cope with the extremely harsh conditions (Lyman et al. 1982). Hibernation, characterized by inactivity of the animal with a general depression of metabolic rate, body temperature (Tb), and neuronal activity, is a very successful adaptation in behavioral, ecological, physiological, and biochemical aspects. Although some invertebrates, amphibians, and reptiles also exploit torpor for escaping unfavourable circumstances (Kayser 1961), torpor and hibernation in birds and mammals are totally different from that of the ectotherms. The Tb of a hibernating bird or mammal is always controlled within a degree or a few degrees above the environmental temperature and never drops below a critical temperature (Wang 1988) whereas the Tb of a torpid ectotherm is essentially the same as the environmental temperature. Additionally, the hibernating bird or mammal can spontaneously arouse from hibernation using exclusive endogenous thermogenesis (Lyman et al. 1982). In contrast, the torpid ectotherm has to be warmed up by exogenous heat. Therefore, hibernation of birds and mammals is an actively controlled, modified form of thermoregulation. In this thesis, mammalian hibernation was studied using ground squirrels. Thus, unless otherwise specified, "hibernation" and "hibernator" used in this thesis refer to mammalian hibernation and mammalian hibernator, respectively.

Among mammals, members from at least six orders are capable of exhibiting torpor or hibernation (Lyman et al. 1982). They can be classified into two groups according to their torpid pattern: non-seasonal and seasonal. Non-seasonal torpidity is largely entrained by food availability, environmental temperature, photoperiod, and/or other external factors (Kilduff 1987). Seasonal torpidity, on the other hand, like hibernation and aestivation, occurs regularly in a certain season of each year and is prominently controlled by an endogenous circannual rhythm (Kilduff 1987, Wang 1989). For seasonal hibernators, such as the ground squirrels used in the present experiments, there are two distinct physiological states within each year: a non-hibernating season and a hibernating season. In non-hibernating season, animals are active and their Tb is maintained relatively constant at around 37°C. The body weight of animals is low and relative stable. When they are exposed to cold in this season, they will maintain their Tb and combat cold through an increase of metabolic rate (Wang 1988) similar to other mammalian non-hibernators. At the onset of the hibernating season, food consumption of the animals increases drastically, which causes a rapid increase in body weight -- doubled in about a month -- to build up body fat (Mrosovsky 1983, Wang 1988). Once the animals store enough energy by fattening, they become anorexic and their body weight declines gradually. If the animals are transferred to a cold environment at this time, they will probably initiate hibernation (Wang 1988). Other than the obvious changes in body weight prior to and/or during hibernation, many invisible, but important, physiological changes also take place in preparing the animals for the prolonged hypometabolic and hypothermic state typical of hibernation. These alterations include modification of enzyme activities (Augee et al. 1984, Pehowich and Wang 1984, 1987, Storey 1989), possibly via phosphorylation (Storey and Storey 1990), hyperinsulinemia and hypertriacylglycerolemia (Florant and Greenwood 1986), lipogenesis (Castex and Sutter 1981, Melnyk et al. 1983), metabolic fuel utilization (Dark et al. 1989, Krilowize 1985, Zimmerman 1982), the ability of regulating intracellular free calcium (Belke et al. 1987, Liu 1993), and endocrine functions (Wang 1982). Therefore, hibernation is a very actively controlled and complex process rather than a simple passive abandonment of homeothermism.

In the hibernation season, however, the animals are not in continuous hypothermia. They invariably arouse periodically to restore euthermia for a few hours to a few days before returning to hibernation again. Thus, a hibernation season consists of many individual hibernation bouts interrupted by periodical arousal and inter-hibernation euthermia (Wang 1979). One hibernation bout refers to the episodes of entry into, maintenance of, and arousal from hibernation.

At entry into hibernation, the heart rate of the animal decreases significantly, with characteristic arrhythmia due to an increase in parasympathetic activity (Lyman et al. 1982). Respiratory rate also decreases, with intermittent apnoea (Lyman et al. 1982). The hypothalamic set-point for Tb regulation declines gradually, promoting a reduction in regulatory thermogenesis and a fall in Tb. When the decline of Tb is faster than that of the set-point, the animal starts to shiver and the metabolic rate increases to slow down the too rapid a decrease in Tb (Hudson 1973). When the animal is in deep hibernation, with Tb as low as -2 to 2°C (Wang 1988, Barnes 1989), its physiological functions are at the minimum: the heart rate is about 5 to 10 beats per minute, which is less than 1/30 of the euthermic value; respiratory rate averages 0.4 times per minute (e.g. golden mantled ground squirrel, Lyman et al. 1982), about 1/90 of the active level; and metabolic rate is less than 1/100 of the euthermic level (Wang 1988). During hibernation, there is also an overall respiratory acidification, with the lowest pH (6.8) observed in the brain (Wang 1989). This acidosis is responsible for the further inhibition of metabolic rate during hibernation (Malan 1986, Bharma and Milsom 1993). Despite the low energy consumption and Tb, the animal is still capable of regulating a new steady-state homeostasis of their internal environment and can respond appropriately to both internal and external disturbances (Kayser 1961, Wang 1989). Upon external or internal stimulus, bursts of muscle action potential appear with an increase in respiratory, heart, and metabolic rates (Lyman et al. 1982). If the stimulus is strong enough, arousal will be initiated. At the commencement of arousal, sympathetic drive is at its maximum. A rapid increase of oxygen consumption results in a gradual increase of Tb, consequent to heat generated by non-shivering and shivering thermogenesis (Lyman et el. 1982, Wang 1989). The acidosis is reversed by hyperventilation at the beginning of arousal (Wang 1988). The anterior portion of the body warms up faster than the posterior end until euthermia is completed restored (Lyman et al. 1982). After hours or days in inter-bout euthermia, the animal re-enters another hibernation bout. The hibernation bouts are repeated until the end of the hibernation season (Wang 1979). The energy saved through the whole hibernation season could be as high as 87.8% (Wang 1979) comparing with the same animal resting in euthermia at the same environmental temperature for the same duration. Overall, the whole annual hibernation cycle is apparently controlled precisely by an endogenous circannual rhythm for the purpose of energy conservation when the animal is confronted by seasonal cold and food shortage.

Since hibernation is a general depression of autonomic functions and an extended form of thermoregulation, appropriate control of the readjusted physiological functions is essential for survival of the animals. The hypothalamic region integrates autonomic functions of the brainstem and controls the release of pituitary hormones (Angevine and Cotman 1981) and is therefore crucial for the maintenance of physiological homeostasis (Heller 1979, Cohen and Shermen 1983). The septum, having numerous efferent and afferent communications with the telencephalic, diencephalic, and mesencephalic nuclei (Hebel and Stromberg 1986, Staiger and Nurnberger 1989, 1991a, b), serves as a relay station between the hypothalamus and the brainstem reticular formation (BSRF) to integrate the somatic and autonomic activities (Swanson 1977, Vinogradova and Brazhnik 1977, Swanson 1987). Medial septal neurons generate rhythmic bursts to entrain the hippocampal theta rhythm (Green and Arduini 1954, Petsche et al. 1962, Stewart and Fox 1990, Tranb and Miles 1991, Smythe et al. 1992), via cholinergic, GABAergic, and possibly monoaminergic projections (Lynch et al. 1977, Storm-Mathisen 1977, Lee et al. 1994). The hippocampus, on the other hand, projects to the lateral septum, which in turn, sends efferents to the medial septum, lateral hypothalamus, and the mamilliary complex (Swanson 1977, Hebel and Stromberg 1986). Other direct or indirect projections from the hippocampus are to the anterior thalamus, the ventromedial and arcuate nuclei of hypothalamus, habenular, and interpeduncular nuclei (Swanson 1977, Hebel and Stromberg 1986). The intense neural connection of the septo-hippocampal complex to the hypothalamic and brain stem regions suggests its important role in physiological regulation. It has been demonstrated that the septo-hippocampal complex is involved in thermoregulation (Murakami et al. 1984, Boulant et al. 1989, Lee et al. 1989, Zeisberger 1990), food intake (King and Nance 1986, Stanley et al. 1989), cardiovascular functions (Wang and Ingenito 1992, 1994), and respiratory suppression (Rector et al. 1993).

During hibernation, neuronal activities (Strumwasser 1959), metabolism (Kilduff et al. 1986, 1989), and gene expression (Bitting et al. 1994, Kilduff et al. 1993) persist in the limbic regions, sometimes even more intense than in the non-hibernating state. Additionally, these nuclei are able to respond to thermal and neurochemical stimuli in the hibernating animals, indicating maintenance of their physiological functions during hibernation despite the drastic decrease in Tb (Beckman and Satinoff 1972, Stanton et al. 1980, Strumwasser 1959, Wunnenberger et al. 1978, Yu and Cai 1993). It is, thus, hypothesized that inhibitory activity of the septo-hippocampal complex to the BSRF is important for transition from one state to the other in a hibernation bout (Heller 1979, Beckman and Stanton 1982, Belousov 1993). More specifically, inhibitory influence from the septo-hippocampal complex to the BSRF is a prerequisite for entry into hibernation (Heller 1979), whereas reversal of this inhibition in the septo-hippocampal region induces arousal of animals from hibernation (Beckman and Stanton 1982, Belousov and Belousova 1992, Belousov 1993). Therefore, the septo-hippocampal complex appears to be an important gating mechanism in determining activity of the hypothalamus and BSRF for the onset and/or termination of hibernation.

Numerous studies have indicated changes in the activities of various neurochemicals during different phases of a hibernation cycle (Glass and Wang 1979, Beckman 1978, Beckman and Stanton 1982, Stanton et al. 1981). In addition, manipulation of their activities modulates hibernation patterns. Thus, these neurochemicals have been postulated to regulate different phases of a hibernation cycle. Opioids represent one group of such neurochemicals speculated to be an important component in controlling the entry and maintenance of hibernation (for review see Wang 1993).

To date, at least five different types of opioid receptors, namely μ, δ, κ, σ, and ϵ, have been proposed based on ligand binding, biological assay, and behavioural effects (Gilbert and Martin 1976, Martin et al. 1976, Lord et al. 1977, Wuster et al. 1979, Zukin and Zukin 1981, 1984). However, only three main types, μ, δ, and κ receptors, have been thoroughly investigated and well defined (Simon 1991, Loh and Smith 1990, Ueda et al. 1991, Barnard and Simon 1993, Reisine and Bell 1993). The μ receptor is termed from morphine-mediated effects. Its selective agonists are [D-Ala^2,N-Me-Phe^4,Gly-ol^5]enkephalin (DAGO) and [N-MePhe^3,DPro^4]morphiceptin (PL017). The δ receptor is named as it was first found prominently in the mouse vas deferens. The selective agonists for δ receptors are [D-Pen^2,D-Pen^5]enkephalin (DPDPE), [D-Ser^2,Leu^5]enkephalin (DSLET), and [D-Ala^2,D-Lue^5]enkephalin (DADLE). The κ receptor is the subtype which mediates the effects of ketocyclazocine; its specific agonists are trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate (U50488), ethyl-ketocyclazocine (EKC), and dynorphin A.

Endogenous opioids, on the other hand, are generated from three distinct gene families: proopiomelanocortin (POMC), proenkephalin, and prodynorphin (Cuello

1983, Khachaturian et al. 1985, Civelli et al. 1986). β-Endorphin is the opioid product of the POMC gene and is a potent agonist at both μ and δ receptors. Non-opioid peptides co-expressed in the POMC family are ACTH and γ-LPH. Met-enkephalin and leu-enkephalin are the post-translational products of the proenkephalin gene. Both of them prefer δ receptors. Dynorphin A 1-17, 1-13, 1-8, along with dynorphin B and neo-endorphin, are post-translational products of the prodynorphin family. Most of the dynorphins have κ preference; the exception is dynorphin A 1-8 which retains δ capability and dynorphin A 1-13 which has high potency to both κ and μ receptors (Akil et al. 1984, 1988, Brush and Shain 1989, Simon 1991).

The diverse endogenous opioids and the three types of opioid receptor are distributed widely in the central nervous systems (CNS), peripheral nervous systems (PNS), and several organs or organ systems (Cuello 1983, Akil et al. 1984, Stengard-Pedersen 1989, Lutz and Pfister 1992). They have been shown to play multi-functional roles in various physiological, pharmacological, and behavioral responses by acting on different types of opioid receptors in different loci (Snyder 1986, Brush and Shain 1989). It has been shown that: (1) intracerebroventricular (icv) or intrahypothalamic administration of dynorphin or other μ opioid agonists can stimulate food consumption at low dose, causing an increase in body weight (Baile et. al 1986, Della-Fera et al. 1990, Shaw et al. 1990, Nizielski et al. 1986). Microinjection studies indicated that all three types of opioid agonists can significantly induce feeding in rats when applied to the specific hypothalamic nuclei (e.g. paraventricular nucleus of the hypothalamus, dorsomedial nucleus of the hypothalamus, and ventromedial nucleus of the hypothalamus) or extra-hypothalamic regions (e.g. the septum, hippocampus, and amygdala; Stanley et al. 1989); (2) opioids modulate heart rate, blood pressure, and respiration, depending on the routes of administration and the type of opioids involved (Akil et al. 1984, Kunos et al. 1988, Olson et. al 1989, 1994). Generally, peripheral κ opioids reduce heart rate and κ and δ opioids inhibit vasoconstriction (Feuerstein and Siren 1987, Illes 1989, Yeadon and Kitchen 1989). Moreover, μ agonists mediate respiratory depression by activating centrally and/or peripherally located μ receptors (Yeadon and Kitchen 1989); (3) opioids have been shown to be involved in thermoregulation (Wang et al. 1987, Adler et al. 1988, 1992, Lee et al. 1989, Burks 1991). It has been shown that κ agonists induce hypothermic responses at high doses both centrally and peripherally (Adler et al. 1988, 1991), whereas μ agonists are mainly responsible for hyperthermia (Spencer et al 1990, Burks 1991, Adler et al. 1992); and (4) opioid peptides have been demonstrated to modulate the activity of other neurotransmitters and/or hormones (Illes 1989). The complex physiological functions of the opioids are quite possibly carried out by modulating other chemical factors. The similarities between the physiological effects of opioids on feeding, the cardiovascular system, respiration, and thermoregulation and those physiological changes prior to and during hibernation prompt the suggestion that certain functional relationships between opioids and hibernation could exist (Wang 1993).

Experimental evidence for the involvement of opioids in hibernation includes: (1) an increase in dynorphin immunoreactivity in the hypothalamus, striatum, and cortex in the hypophagic phase of the annual hibernation cycle (Nizielski et al. 1986). The sensitivity to manipulation of feeding by opioid agonists or antagonists is different in the hyperphagic vs. the hypophagic phases of the annual hibernation cycle (Nizielski et al. 1986), suggesting a seasonal alteration in the content of endogenous opioids and responsiveness to exogenous opioids; (2) administration of the non-selective opioid antagonist naloxone (Margules 1979, Beckman and Liados-Eckman 1985), naltrexone (Kromer 1980), the κ antagonist nor-binaltorphine (nor-BNI, Yu and Cai 1993), the δ antagonist naltrindole (Yu and Cai 1993) and the opioid agonists met-enkephalin (Beckman et al. 1992) or β-endorphin (Beckman et al. 1993a) to hibernating animals caused premature arousal from hibernation or shortening of the hibernating bouts, which indicates the necessity of specific opioid activity for the maintenance of hibernation; (3) an increase in met-enkephalin-like substances in brain extracts (Kramarova et al. 1983) and met-enkephalin immunoreactivity in perikarya of the septum and hypothalamus of the hibernating animals (Nurnberger et al. 1991) have been reported; (4) failure in the development of physical dependence to morphine in the hibernating state (Beckman et al. 1981), but not when the animals are in an induced general CNS depression by pentobarbitol (Beckman et al. 1993b), indicating the reduction in responsiveness of opioid receptor to exogenous morphine is state-dependent; (5) a significant reduction in μ opioid agonist dihydromorphine binding in the hippocampus and septum during hibernation (Beckman et al. 1986) and a decrease in κ binding in the brain homogenate of hibernating animals (Aloia et al. 1985), indicating a state-dependent down-regulation of selective opioid receptors; and (6) a decrease in thermoregulatory response to administration of exogenous opioids in the hibernating phase (Wang et al 1987, Lee et al. 1989), suggesting desensitization or down-regulation of specific opioid receptors by increased activity of corresponding endogenous opioids. Taken together, these studies tend to suggest that an increase in endogenous opioid activity may be a main characteristic of the hibernation season and in the hibernating state. Particularly, endogenous opioids may regulate the entry into and/or maintenance of hibernation. However, the exact mechanisms, what opioid types and in which specific regions, the opioids regulate hibernation still remain unknown.

To date, only limited number of studies on the changes in opioid activity during the hibernation cycle have been carried out. Most of them mainly focused on the changes in one type of opioid or receptor (Beckman et al. 1986), in whole brain homogenates (Aloia et al. 1985, Bourhim et al. 1993), or in selected CNS regions (Wang et al. 1987, Kolaeva et al. 1989, Lee et al. 1989, Nurnberger et al. 1991). No systematic investigation has been conducted to examine the changes of each endogenous opioid and opioid receptor type in a seasonal hibernation cycle.

Based on the above mentioned model of CNS regulation of hibernation (Heller 1979, Beckman and Stanton 1982, Belousov 1993), the present study is mainly focused on changes of opioid activity in the septo-hippocampal complex to provide further understanding on the differential roles of various endogenous opioids in regulating the hibernation cycle. The main objectives are: (1) to substantiate our existing hypothesis that: "a differential reduction of receptor responsiveness to exogenous opioids and receptor binding in specific CNS regions is associated with the hibernating state." A detailed documentation will be provided on the seasonal alteration of three types of opioid receptors in the regulation of thermoregulatory response, the modulation of 5-HT (serotonin) activity, and their receptor binding characteristics; (2) to validate our supposition that different central and peripheral opioids are involved in regulating different phases of a hibernation cycle by comparing the regional (CNS and peripheral) differences in the content of each endogenous opioids in a hibernation cycle; and (3) to test the hypothesis that: "the functional role of endogenous opioids in regulating hibernation should be reflected by their seasonal differences in activity levels in selective CNS sites." To answer this, *in vivo* microdialysis will be used to compare the apparent turnover of various opioids under different phases of a hibernation cycle to provide a functional assessment of the endogenous opioid activity. When all the results are combined from the above mentioned approaches, a tentative model or scheme on how endogenous opioids may be functionally involved in the regulation of seasonal hibernation will be presented.

References

Adler M. W., Geller E. B., Rosow C. E., and Cochin J. (1988) The opioid system and temperature regulation. Ann Rev Pharmacol Toxicol 28:429-49

Adler M.W., Bradley E., Martinez R., and Geller E.B. (1991) Production of hypothermia in teh guinea pig by a kappa-agonist opioid alone and in combination with chlorpromazine. Pharmacol Biochem Behav 40:129-132

Adler M.W. and Geller E.B. (1992) Physiological functions of opioids: temperature regualtion. Handbook of experimental pharmacology. Vol. 104/II Opioids II Herz A., Akil H., and Simon E.J. (eds), pp 205-238, Pergamon Press, New York.

Akil H., Watson S. T., Yang E., Lewis M. E., Khaehaturian H. and Waller J. M. (1984) Endogenous opioids: biology and function. Ann Rev Neurosci 7:223-255

Akil H., Bronstein D., and Mansour A. (1988) Overview of the endogenous opioid systems: anatomical, biochemical and functional issues. Endorphins, Opiates and Behavioral Processes Rodgers R.J. and Cooper S.J. (eds), pp 1-23, Chichester, New York.

Aloia R.C., Vasquez B.J., and Tucker S.L. (1985) Opioid receptors in the brain of active and hibernating ground squirrels, *Spermophilus lateralis*. Abstracts for Living in the Cold (Fallen Leaf Lake, Carlifornia) Heller H.G., Musacchia X.J., and Wang L.C.H. (eds), p 211

Angevine J.B. and Cotman C.W. (1981) Principles of neuroanatomy Oxford University Press, New York.

Augee M.L., Pehowich D.J., Raison J.K., and Wang L.C.H. (1984) Seasonal and temperature related changes in mitochondrial membranes associated with torpor in the mammalian hibernator *Spermophilus richardsonii*. Biochim Biophys Acta 776:27-36

Baile C. A., Melaughlin C. L. and Della-Fera M. A. (1986) Role of cholecystokinin and opioid peptides in control of food intake. Physiol Rev 66:172-234

Barnard E. A. and Simon J. (1993) Opioid receptors. Neurotransmitter Receptors Hucho F. (ed), pp 297-323, Elsevier, Amsterdam.

Barnes B.M. (1989) Freeze avoidance in a mammal: body temperatures below

0°C in an arctic hibernator. Science 244:1593-1595

Beckman A.L. and Satinoff E. (1972) Arousal from hibernation by intrahypothalamic injections of biogenic amines in ground squirrels. Am J Physiol 222:875-879

Beckman A.L. (1978) Hypothalamic and midbrain function during hibernation. Current studies of hypothalamic function Vol.2, Veale W.L. and Lederis K. (eds), pp 29-43, Karger, Basel.

Beckman A. L., Llados-Eckman C., Stanton T. L., and Adler M. W. (1981) Physical dependence on morphine fails to develop during the hibernating state. Science 212:527-529

Beckman A.L. and Stanton T.L. (1982) Properties of the CNS during the state of hibernation. The neural basis of behaviour Beckman A.L. (ed), pp 19-45, Spectrum, New York.

Beckman A. L. and Llados-Eckman C. (1985) Antagonism of brain opioid peptide action reduces hibernation bout duration. Brain Res 328:201-205

Beckman A., Dean R. R., and Wamsley J. K. (1986) Hippocampal and cortical opioid receptor binding: changes related to the hibernation state. Brain Res 386:223-231

Beckman A.L., Liados-Eckman C., and Stanton T.L. (1992) Reduction of hibernation bout by intraventricular infusion of met-enkephalin. Brain Res 588:159-163

Beckman A., Kim M.M., and Newman J.R. (1993a) β-Endorphin Action during Hibernation: Dose-dependent Reduction of Bout Length Produced by Intracerebroventricular Infusion in Ground Squirrels (*Citellus Lateralis*). Living In the Cold III. p. 4

Beckman A.L., Beaver T.A., and Lewis F.A. (1993b) Morphine physical dependence in the hibernator: cental nervous system mechanisms underlying the development of dependence remain functional during depression induced by pentobarbital anaesthesia. Life Sci 52:1079-1086

Belke D.D., Pehiowich D.J., and Wang L.C.H. (1987) Seasonal variation in calcium uptake by cardiac sarcoplasmic reticulum in a hibernator, the Richardson's ground squirrel. J Therm Biol 12:53-56

Belousov A.B. and Belousova J.V. (1992) State-dependent and state-independent excitibility of the medial septal neurons in brain slices of ground squirrel, *Citellus undulatus*. Neurosci 38:599-608

Belousov A.B. (1993) On possible mechanisms of the septohippocampal system participation in the neural control of hibernation. Life in the cold: Ecological, physiological, and molecular mechanisms Cynthia C., Florant G.L., Wunder B.A., and Horwitz B. (eds), pp 291-296, Westview Press, Colorado.

Bharma S. and Milsom W.K. (1993) Acidosis and metabolic rate in golden mantled ground squirrels (*Spermophilus lateralis*). Respiration Physiol 94:337-51

Bitting L., Sutin E.L., Watson F.L., Learal L.E., O'ttara B.F., Heller H.L., and Kilduff T.S. (1994) c-fos mRNA increases in the ground squirrel suprachiasmatic nucleus during arousal from hibernation. Neurosci Lett 165:117-21

Boulant J.A., Curras M.C., and Dean J.B. (1989) Neurophysiological aspects of thermoregulation. Advances in comparative and environmental phyisology Vol.4 Wang L.C.H. (ed), pp 117-160, Springer-Verlag, Berlin.

Bourhim N., Elkebbaj M.S., Ouafik L.H., Chautard T.H., Giraud P., and Oliver C. (1993) Opioid binding sites in Jerboa (*Jaculus orientalis*) brain: a biochemical comparative study in teh awake-active and induced hibernating states. Comp Biochem Physiol 104B:607-615

Brush F.R. and Shain C.N. (1989) Endogenous opioids and behavior. Psychoendocrinology Brush F.R. and Levine S. (eds), pp 379-435, Academic Press, San Diego.

Burks T.F. (1991) Opioids and opioid receptors in thermoregulation. Thermoregulation: Pathology, Pharmacology and Therapy. Schonbaum E. and Lomax P. (eds), pp 489-508, Pergamon Press, New York.

Castex C. and Sutter B.C.J. (1981) Insulin binding and glucose oxidation in edibble dormouse (*Glis glis*) adipose tissue: seasonal variations. Gen Comp Endocrinol 45:273-278

Civelli O., Douglass J., Rosen H., Martens G., and Herbert E. (1986) Biosynthesis of opioid peptides. Opioid Peptides: Molecular Pharmacology, Biosynthesis, and Analysis. NIDA Monograph Series 70:21-42, NIDA Publication, Rockville.

Cohen D.H. and Sherman S.M. (1983) The autonomic nervous system and its central control Physiology Berne R.M. and Levy M.N. (eds), pp 314-335, C V Mosby, St. Louis.

Cuello A.C. (1983) Central distribution of opioid peptides. Med Bull 39:11-16

Dark J., Stern J.S., and Zucker I. (1989) Adipose tissue dynamics during cyclic weight loss and weight gain of ground squirrels. Am J Physiol 256:R1286-R1292

Della-Fera M. A., Baile C. A., Coleman B. D., Miner J. L., and Pacerson J. A. (1990) Nervous system injection of dynorphin-(1-13) overrides gastric satiety factors in sheep. Am J Physiol 258:R946-R950

Feuerstein G. and Siren A.L. (1987) The opioid peptides: a role in hypertension? Hypertension 9:561-565

Florant G. and Greenwood M.R.C. (1986) Seasonal variations in pancreatic function in marmots: the role of pancreatic hormones and lipoprotein lipase in fat deposition. Living in the Cold. Heller H.C., Musacchia X.J., and Wang L.C.H. (eds), pp 273-280, Elsevier, Amsterdam.

Gilbert P.E., and Martin W.R. (1976) The effects of morphine- and nalorphine-like drgus in the nondependent, morphine-dependent and cyclazocine-dependent chronic spinal dog. J Pharmacol Exp Ther 198:66-82

Glass J.D. and Wang L.C.H. (1979) Thermoregulatory effects of intracerebroventricular injection of serotonin and a monoamine oxidase inhibitor in a hibernator, *Spermophilus richardsonii.* J Therm Biol 4:149-156

Green J.D. and Arduini A.A. (1954) Hippocampal electrical activity in arousal. J Neurophysiol 17:533-557

Hebel R. and Stromberg M.W. (1986) Anatomy and Embryology of the Laboratory Rat BioMed Verlag, Worthsee.

Heller H. C. (1979) Hibernation: neural aspects. Ann Rev Physiol 41:305-321

Hudson J.W. (1973) Torpidity in mammals. Comparative physiology of thermoregulation Vol.3, Whittow G.C. (ed), pp 97-165, Academic Press, London.

Illes P. (1989) Modulation of transmitter and hormone release by multiple neuronal opioid receptors. Rev Physiol Biochem Pharmacol 112:141-233

Kayser C. (1961) The physiology of nature hibernation Pergamon Press, New York.

Khachaturian H., Lewis M.E., Schafer M.K.-H., and Watson S.J. (1985) Anatomy of the CNS opioid system. TINS 8:111-119

Kilduff T.S. (1987) Hibernation. Encyclopedia of Neuroscience. Vol.1, Adelman G. (ed), pp 486-488, Birkhaeuser, Boston.

Kilduff T. S., Radlke C. D., and Heller H. C. (1986) Neural activity during mammalian hibernation. Living in the Cold Heller H.C., Musacchia X.J., and Wang L.C.H. (eds), pp 215-223, Elsevier, Amsterdam.

Kilduff T. S. and Heller H. C. (1989) Neurochemical studies of hibernation. Living in the Cold II Malan A. and Canguilhem B. (eds), pp 467-475, John Libbey Eurotext, Montrouge.

Kilduff T.S., O'Hara B.F., Sutin E.L., Watson F.L., Bitting L., and Heller H.C. (1993) Immediate early gene expression in brain across the hibernation cycle. Life in the cold: Ecological, physiological, and molecular mechanisms Cynthia C., Florant G.L., Wunder B.A., and Horwitz B. (eds), pp 305-314, Westveiw Press, Colorado.

King T.R. and Nance D.M. (1986) Differential effects of neurotoxic lesions on psychoneuroendocrine functions. Pharmacol Biochem Behav 24:107-114

Kolaeva S.G., Kramarova L.I., and Yarying K.N. (1989) The role of opioids and TRH in the hibernation bout/arousal cycle of ground squirrels *Citellus undulatus*. Living in the cold II Malan A. and Canguilhem B. (eds), pp 461-466, John Libbey Eurotext, Montrouge.

Kramarova L.I., Kolaeva S.G., Yukhanaov R.Y., and Rozhanets V.V. (1983) Content of DSIP, enkephalins, and ACTH in some tissue of active and hibernating ground squirrels. Comp Biochem Physiol 74C:31-33

Krilowize B.L. (1985) Ketone body metabolism in a ground squirrel during hibernation and fasting. Am J Physiol 249:R462-470

Kromer W. (1980) Naltrexone influence on hibernation. Experientia 36:581-582

Kunos G., Mosqueda-Garcia R., and Mastrianni J. A. (1988) Endophinergic neurons in the brainstem and the control of blood pressure and heart rate.

Regulatory Roles of Opioid Peptides Illes P. and Farsang C. (eds), pp 460-470, VCH, Weinheim.

Lee M.G., Chrobak J.J, Sik A., Wiley R.G., and Buzsaki G. (1994) Hippocampal theta activity following selective lesion of the septal cholinergic system. Neuroscience 62:1033-1047

Lee T. F., Nurnberger F., Jourdan M. L. and Wang L. C. H. (1989) Possible involvement of septum in seasonal changes in thermoregulatory responses to met-enkephalinamide in ground squirrels. Thermoregulation: Research and clinical applications Schonbaum E. and Lomax P. (eds), pp 200-203, Kruger, Basel.

Loh H.H and Smith A.P. (1990) Molecular characterization of opioid receptors. Annu Rev Pharmacol Toxicol 30:123-147

Lord J.A.H., Waterfield A.A, Hughes J., and Kosterlitz H.W. (1977) Endogenous opioid peptides: mutiple agonists and rceptors. Nature 267:495-499

Liu B. (1993) Cardiac function and regulation of intracellular Ca^+ at low temperature in hibernating and non-hibernating mammals. (Ph.D. Thesis, University of Alberta)

Lutz R.A. and Pfister H.P. (1992) Opioid receptors and their pharmacological profiles. J Receptor Res 12(3):267-286

Lyman C.P., Wills J.S., Malan A., and Wang L.C.H. (eds) (1982) Hibernation and torpor in mammals and birds Academic Press, New York.

Lynch G.S., Rose G., and Gall C.M. (1977) Anatomical and functional aspects of the septo-hippocampal projections. Functions of the Septo-Hippocampal System Ciba Foundation Symposium, 58, pp 5-19, Elsevier, Amsterdam.

Malan A. (1986) pH as a control factor in hibernation. Living in the Cold Heller H.C., Musacchia X.J., and Wang L.C.H. (eds), pp 61-70, Elsevier, Amsterdam.

Margules D. L., Goldman B., and Finck A. (1979) Hibernation: an opioid-dependent state? Brain Res Bull 4:721-724

Martin W.R., Eades C.G., Thompson J.A., Huppler R.E., and Gilbert P.E. (1976) The effects of morphine- and nalorphine-like gruds in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther 197:518-532

Melnyk R.B., Mrosovaky N., and Martin J.M. (1983) Spontaneous obesity and weight loss: insulin binding and lipogenesis in the dormouse. Am J Physiol 245:R403-R407

Mrosovsky N. (1983) Cyclical obesity in hibernators: the search for the adjustable regulator. Recent advances in obesity research Vol.4, Hirsh J. and Itallie T.B. (eds), pp 45-56, Libey, London.

Murakami N., Morimoto A., and Watanabe T. (1984) Radioactive deoxyglucose uptake in the rat CNS during thermoregulatory responses. Thermal Physiology Hales J.R.S. (ed) pp. 105-108, Raven Press, New York.

Nizielski S.E., Levine A.S., Morley J.E., Hall K.A., and Gosnell B.A. (1986) Seasonal variation in opioid modulation of feeding in the 13-lined ground squirrel. Physiol Behav 37:5-9

Nurnberger F, Lee T. F., Jourdan M. L., and Wang L. C. H. (1991) Seasonal changes in methionine-enkephalin imµnoreactivity in the brain of hibernator, *Spermophilus columbianus.* Brain Res 547:115-121

Olson G. A., Olson R. O., and Kastin A. J. (1989) Endogenous opiates: 1987. Peptides 10:205-236

Olson G.A., Olson R.D., and Kastin A.J. (1994) Endogenous opiates: 1993. Peptides 15:1513-1556

Pehowich D.J. and Wang L.C.H. (1984) Seasonal changes in mitochondrial succinate dehydrogenase activity in a hibernator, *Spermophilus richardsonii.* J Comp Physiol B Biochem Syst Environ Physiol 154:495-501

Pehowich D.J. and Wang L.C.H. (1987) Stoichiometry of H^+ efflux to respiration-dependent Ca^{2+} up-take and oxygen consumption in liver mitochondria from a hibernator. Physiol Zool 60:114-120

Petsche H., Stumpf C.H., and Gogolak G. (1962) The significance of the rabbits septum as a relay station between the midbrain and the hippocampus I. the control of hippocampus arousal activity by the septum cells. EEG Clin Neurophysiol 14:202-211

Rector D.M., Poe G.R., and Harper R.M. (1993) Imaging of hippocampal and neocortical neural activity following intravenous cocine adminstration in freely behaving rats. Neuroscience 54:663-641

Reisine T. and Bell G.I. (1993) Molecular biology of opioid receptors. TINS 16:506-510

Shaw W. N., Mitch C. H., Leander J. D., and Zimmerman D. M. (1990) Effect of phenylpiperidine opioid antagonists on food consumption and weight gain of the obese zucker rat. J Pharmacol Exp Ther 253(2):85-89

Simon E.J. (1991) Opioid receptors and endogenous opioid peptides. Med Res Rev 11:357-374

Smythe J.W., Colom L.V., and Bland B.H. (1992) The extrinsic modulation of hippocampal theta depends on the coactivation of cholinergic and GABA-ergic medial septal inputs. [Review] Neurosci Biobehav Rev 16:289-308

Snyder S.H. (1986) Neuronal receptors. Ann Rev Physiol 48:461-471

Spencer R.L., Hruby V.J, and Burks T.F. (1990) Alteration of thermoregulatory set point with opioid agonists. J Pharmacol Exp Ther 252:696-705

Staiger J.F. and Nurnberger F. (1989) Pattern of afferents to the lateral septum in the guinea pig. Cell Tissue Res 257:471-490

Staiger J.F. and Nurnberger F. (1991a) The efferent connections of the lateral septal nucleus in the guinea pig: projections to the diencephalon and brainstem. Cell Tissue Res 264:391-413

Staiger J.F. and Nurnberger F. (1991b) The efferent coonections of the lateral septal nucleus in the guinea pig: intrinsic coonectivity of the septum and projections to other telencephalic areas. Cell Tissue Res 264:415-426

Stanton T.L., Winokur A., and Beckman A.L. (1980) Reversal of natural CNS depressionby TRH action in thre hippocampus. Brain Res 181:470-475

Stanton T.L., Beckman A.L., and Winokur A. (1981) Thyrotropin-releasing effects in the central nervous system: dependence on arousal state. Science 214:678-681

Stanley B.G., Lanthier D., and Leibowitz S.H. (1989) Multiple brain sites sensitive to feeding stimulation by opioid agonists: a cannula-mapping study. Pharmacol Biochem Behav 31:825-832

Stengaard-Pedersen K. (1989) Opioid Peptides and Receptors Progress in Histochemistry and Cytochemicstry. Vol. 20, No. 3, G Fischer, Stuttgart.

Stewart M. and Fox S.E. (1990) Do septal neurons pace the hippocampal theta rhythm? TINS 13:163-168

Storey K.B. (1989) Integrated control of metabolic rate depression via reversible phosphorylation of enzyme in hibernating mammals. Living in the cold II Malan A. and Canguilhem B. (eds), pp 309-319, John Libbey Eurotext, Montrouge.

Storey K.B. and Storey J.M. (1990) Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and aestivation. Quarterly Rev Biol 65:145-174

Storm-Mathisen J. (1977) Localization of putative transmitters in the hippocampal formation (with a note on the conections to septum and hypothalaums). Functions of the Septo-Hippocampal System Ciba Foundation Symposium 58, pp 49-79, Elsevier, Amsterdam.

Strumwasser F. (1959) Regulatory mechanisms, brain activity and behaviour during deep hibernation in the squirrel, *Citellus beecheyi*. Am J Physiol 196:23-30.

Swanson L.W. (1977) The anatomical organization of septo-hippocampal projections. Functions of the Septo-Hippocampal System Ciba Foundation Symposium 58, pp 25-43, Elsevier, Amsterdam.

Swanson L.W. (1987) The hypothalamus. Handbook of chemical neuroanatomy Vol.5, Bjorklund A., Hokfelt T., and Swanson L.WL. (eds)), pp 125-277, Elsevier, Amsterdam.

Tranb R.D. and Miles R. (1991) Neuronal networks of the hippocampus Cambridge University Press, New York.

Ueda H., Nozaki M., and Satoh M. (1991) Mutiple opioid receptos and GTP-binding proteins. Comp Biochem Physiol 98C:157-169

Vinogradova O.S. and Brazhnik E.S. (1977) Single unit and lesion experiments on the sensory inputs to the hippocampal cognitive map. Functions of the Septo-Hippocampal System Ciba Foundation Symposium 58, pp 145-171, Elsevier, Amsterdam.

Wang J.Q. and Ingenito A.J. (1992) Centrally mediated cardiovascular actions of dynorphin A(1-8) on rat hippocampal formation. J Pharmacol Exp Ther 261:678-685

Wang J.Q. and Ingenito A.J. (1994) Cardiovascular effects of microinjection of dynrophin-A(1-8) into the hippocampus in conscious, spontaneously hypertensive and normotensive Wistar-Kyoto rats. Clin Exp Hypertens 16:229-243

Wang L. C. H. (1979) Time patterns and metabolic rates of natural torpor in the Richardson's ground squirrel. Can J Zool 57:149-155

Wang L.C.H. (1982) Hibernation and the endocrine. Hibernation and topor in mammals and birds Lyman C.P., Willis J.S., Malan A., and Wang L.C.H. (esd), pp 206-236, Academic Press, New York.

Wang L. C. H.(1988) Mammalian hibernation: an escape from the cold. Advances in comparative and environmental physiology Vol.2, Gilles R. (ed), pp 1-45, Springer-Verlag, Berlin.

Wang L.C.H. (1989) Ecological, physiological, and biochemical aspects of torpor in mammals and birds. Advances in comparative and environmental physiology Vol.4, Wang L.C.H. (ed), pp 361-401, Springer-Verlag, Berlin.

Wang L. C. H., Lee T. F., and Jourdan M. L. (1987) Seasonal difference in thermoregulatory responses to opiates in a mammalian hibernator. Pharmacol Biochem Behav 26:565-571

Wang L.C.H. (1993) Is endogenous opioid involved in hibernation? Life in the cold: Ecological, physiological, and molecular mechanisms Cynthia C., Florant G.L., Wunder B.A., and Horwitz B. (eds), pp 297-304, Westview Press, Colorado.

Wunnenberg W., Merker G., and Speulda E. (1978) Thermosensitivity of preoptic neurons and hypothalamic integrative function in hibernators and non-hiberantors. Stratagies in Cold: natural torpidity anf thermogenesis Wang L.C.H. and Hudson J.W. (eds), pp 267-297, Academic Press, London.

Wuster M., Shulz R., and Herz A. (1979) Specificity of opioids towards the *mu-*, *delta-*, and *epsilon-*opiate receptors. Neurosci Lett 15:193-198

Yeadon M. and Kitchen I. (1989) Opioids and respiration. Prog Neurobiol 33:1-16

Yu L.C. and Cai Y.P. (1993) Effects of opioid receptors antagonists administration to suprachiasmatic nucleus on hibernation of ground squirrels *Citellus Dauricus*. Comp Biochem Physiol 104C:249-252

Zeisberger E. (1990) The role of septal peptides in thermoregulation and fever. Thermoreception and Temperature Regulation Bligh J. and Voigt K. (eds), pp 273-283, Springer-Verlag, Berlin.

Zimmerman M.L. (1982) Carbohydrate and torpor duration in hibernating golden-mantled ground squirrels (*Citellus lateralis*). J Comp Physiol 147B:129-135

Zukin R.S. and Zukin S.R. (1981) Multiple opiate receptors: emerging concepts. Life sci 29:2681-2690

Zukin R.S. and Zukin S.R. (1984) The case for multiple opiate receptors. TINS 7:160-164

CHAPTER TWO

In vitro and *in vivo* study on state-dependent changes in opioid-mediated 5-HT release

Introduction

Opioid peptide-containing perikarya and terminals are widely distributed in the central nervous system (CNS) (Angevine and Cotman 1981, Akil et al. 1984, Simon 1991). All three types of opioid receptors, namely μ, δ, and κ, have been located in the CNS. However, each of them distributes in a different pattern (Akil et al. 1988, Sharif and Hughes 1989, Stengaard-Pedersen 1989) and is involved in different physiological functions by modulating the release of other neurotransmitters (Bradley and Brooks 1984, Illes 1989, Simon 1991). For instance, activation of κ receptors inhibits K^+-stimulated acetylcholine (ACh) release from the guinea pig thalamus (Siniscalchi et al. 1990) and the striatum and hippocampus (Izquierdo 1990), whereas μ receptor activation depresses K^+-stimulated ACh release from the rat cerebral cortex (Jhamandas et al. 1975) and the striatum and hippocampus (Izquierdo 1990). Opioids have also been shown to inhibit the K^+- or electric-evoked release of dopamine (Loh et al. 1976), norepinephrine (Taube et al. 1976, Mulder et al. 1989, Simmonds et al. 1992, Matsumoto et al. 1994), and GABA (Cohen et al. 1992) in various regions of rat CNS. In addition, it has been indicated that central or peripheral administration

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This chapter has been published in part as articles entitled "State-dependent variation in the inhibitory effect of [D-Ala^2,D-Leu^5]-enkephalin on hippocampal serotonin release in ground squirrels" in Life Sci 48:175-181 (1991) and "The modulatory effects of *mu* and *kappa* opioid agonists on 5-HT release from hippocampal and hypothalamic slices of euthermic and hibernating ground squirrels" in Life Sci 53:1957-1965 (1993).

of opioid agonists reduces spontaneous secretion of ACh from the rat cerebral cortex (Jhamandas et al. 1977) and alters basal dopamine efflux in several CNS regions (Gudelsky and Porter 1979, Lubetzki et al. 1982, Di Chiara and Imperato 1988). Therefore, opioidergic system seems to impose both tonic and phasic regulation on the secretion of other neurotransmitters.

5-Hydroxytryptamine (5-HT) has been postulated as one of the important neurochemicals in regulating entry into and maintenance of hibernation. It has been demonstrated that 5-HT turnover (Novatona et al. 1975) and the amount of 5-hydroxyindoleacetic acid (5-HIAA, Lin and Pivorun 1989) increased during hibernation or torpor. Recently, an increase in the activity of tryptophan hydroxylase, a 5-HT synthesis enzyme, prior to hibernation has also been observed (Popova et al. 1993). Furthermore, chemical or electrical lesioning to reduce the 5-HT source from the median raphe nuclei has been shown to prevent the onset of hibernation (Spafford and Pengelley 1971; Canguilhem et al. 1986). In addition, opioids can alter 5-HT turnover (Van Loon and Souza 1978, Ahtee and Attila 1980; Forchetti et al. 1982) and modulate 5-HT release (Passarelli and Costa 1988; Yoshioka et al. 1993) in the CNS. Serotonergic terminals, projected from the raphe nuclei, are found to overlap with opioid-containing terminals in many CNS regions (Moore et al. 1978, Azmitia and Segal 1978, Angevine and Cotman 1981, Sharif and Hughes 1989). Functionally, opioids have been reported to interact with the serotonergic system in regulating body temperature (Tb, Oka 1978), pain perception (Ho and Takemori 1989, Millan and Colpaert 1990, Sawynok 1989), hypotension (Berger and Ramire 1988), and feeding (Robert et al. 1989, 1991). However, because of the difference in distribution (Akil et al. 1984, Mansour et al. 1988) and post-receptor events (Ueda et al. 1991, Childers 1991, Barnard and Simon 1993) of different opioid receptors, each receptor type has been demonstrated to mediate different physiological responses (for review see Mansuor et al. 1988, 1994, Wollemann 1993). Thus, it is possible that various opioids could contribute differently to the regulation of hibernation cycles through their differential modulating effects on the CNS serotonergic activity.

Since the septo-hippocampal complex is a very important relay station between the autonomic center, the hypothalamus and brain stem reticular formation (BSRF), and has been demonstrated to be metabolically and electrically active during hibernation, it has been proposed to be a crucial component in regulating the hibernation cycle (Heller 1979, Beckman and Stanton 1982, Belousov 1993, Chapter One). The amount of inhibitory activity from the septo-hippocampal complex to the hypothalamus and BSRF determines the state of the animal in the hibernation cycle. To investigate the possible role of different opioids in regulating the hibernation cycle by differentially modulating 5-HT release, three opioid agonists, [D-Ala^2,N-Me-Phe^4,Gly-ol^5]enkephalin (DAGO), [D-Ala^2,D-Leu^5]enkephalin (DADLE), and trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzenearetamide methanesulfonate hydrate (U50488) (for μ, δ, and κ receptor, respectively), were used in the present study, using an *in vitro* brain slice perifusion for the hippocampal and hypothalamic regions and an *in vivo* microdialysis technique for the septum.

Methods

The experimental protocol has been previously approved by the University of Alberta Animal Use Committee, following the guidelines of the Canadian Council on Animal Care.

Animals Richardson's ground squirrels (*Spermophilus richardsonii*) were live trapped in the area surrounding Edmonton, Alberta and the Columbian ground squirrels (*Spermophilus columbianus*) were trapped in the Rocky Mountains in Alberta. No attempt was made to separate the sexes of either species. Six squirrels were kept together in a group cage at 22°C under natural photoperiod with free access to food (rodent chow, mixed grains, and sunflower seeds) and water. Their body weights were recorded weekly and monitored throughout the year. The transition from the non-hibernation season to the hibernation season was characterized by hyperphagia and a rapid body weight gain followed by weight plateau and anorexia. When their body weights plateaued, the animals were transferred to a cold (4 ± 1°C), dark walk-in environmental chamber to facilitate hibernation. Their activity was checked and recorded daily. The hibernating state was verified as lack of movement, slow and shallow breathing (less than one per minute), and when sawdust placed on the squirrel's back remained on the following day. The hibernating Richardson's ground squirrels used in the brain slice perifusion study were those who had completed at least two hibernation bouts. They were sacrificed while hibernating (Tb=5-7°C). Non-hibernating squirrels were those whose body weight was relatively low and stable for at least two months and were sacrificed while euthermic (Tb=37-38°C). For microdialysis studies, Columbian ground squirrels were cannulated while euthermic between hibernation bouts and used following artificial arousal on the third day of their hibernation bout. Non-hibernating squirrels were used one week after the cannulation.

Brain slice perifusion Hibernating or non-hibernating Richardson's ground squirrels were sacrificed by decapitation. The brain was rapidly removed from the skull and the hypothalamus and hippocampi from both sides of the brain were immediately separated and chopped into slices (300 μm thick) using a McIlwain tissue chopper. The slices were then incubated in oxygenated Krebs' solution (NaCl 117 mM, KCl 3.5 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 28 mM, glucose 11 mM, pH 7.4) containing 0.2 mM ascorbic acid, 1 μM pargyline, and 0.1 μM [^{3}H] 5-HT creatinine sulfate (specific activity 14.5 Ci/mmol), at 37°C for 30 minutes. Following the incubation, the slices were transferred to Brandel (SF-12 multi-channel superfusion system) perifusion chambers (100 µl/ chamber, about 80 mg of wet tissue) and superfused with oxygenated Krebs' solution at a flow rate of 1 ml per minute at 37°C. The first 30 minutes was to wash off external [^{3}H] 5-HT, which has not been taken up into the slices and the perfusate discarded. Starting from minute 31, the eluents were collected every two minutes. The release of 5-HT was stimulated twice, four minutes each time, at minute 46 (S_1) and minute 72 (S_2) using 35 mM K^+ Krebs' solution (increasing KCl to a concentration of total K^+ 35 mM and reducing NaCl to balance ion concentration). Various concentrations (ranging from 10^{-8} to 10^{-5} M) of the opioid agonists (DAGO, DADLE, and U50488) were added to the superfusion medium immediately after the first stimulation (i.e., at minute 50) and remained in the Kreb's solution throughout the rest of the experiment (Figure II.1). At the end of the experiment, the tissue slices were removed and solubilized in 1.0 ml 1 M NaOH. The radioactivity in the slices and the superfusates was determined by liquid scintillation-spectrometry with a LKB 1217 Rackbeta scintillation counter. The amount of [^{3}H] 5-HT released in each fraction was expressed as a percentage of total radioactivity of all superfusion fractions and the tissue slices. The total radioactivity of the first release (S_1), which did not include any opioid agonists, was used as a "self-control". The effect of various opioids on 5-HT release was measured as the ratio of S_2/S_1. Both S_2 and S_1 were first corrected by subtracting baseline radioactivity (average value of the fraction before the release and the one right after the release). The results were normalized using the control group (i.e., no opioid agonist was added throughout the perifusion period) as 100 percent.

In vivo microdialysis Since the septum is a relatively small region, the amount of tissue obtained from one animal is not sufficient to perform a single brain slice perifusion experiment. As it is almost impossible to have two hibernating animals within the same phase of the hibernation cycle, pooling septal samples together from hibernating animals may mask the state-dependent changes.

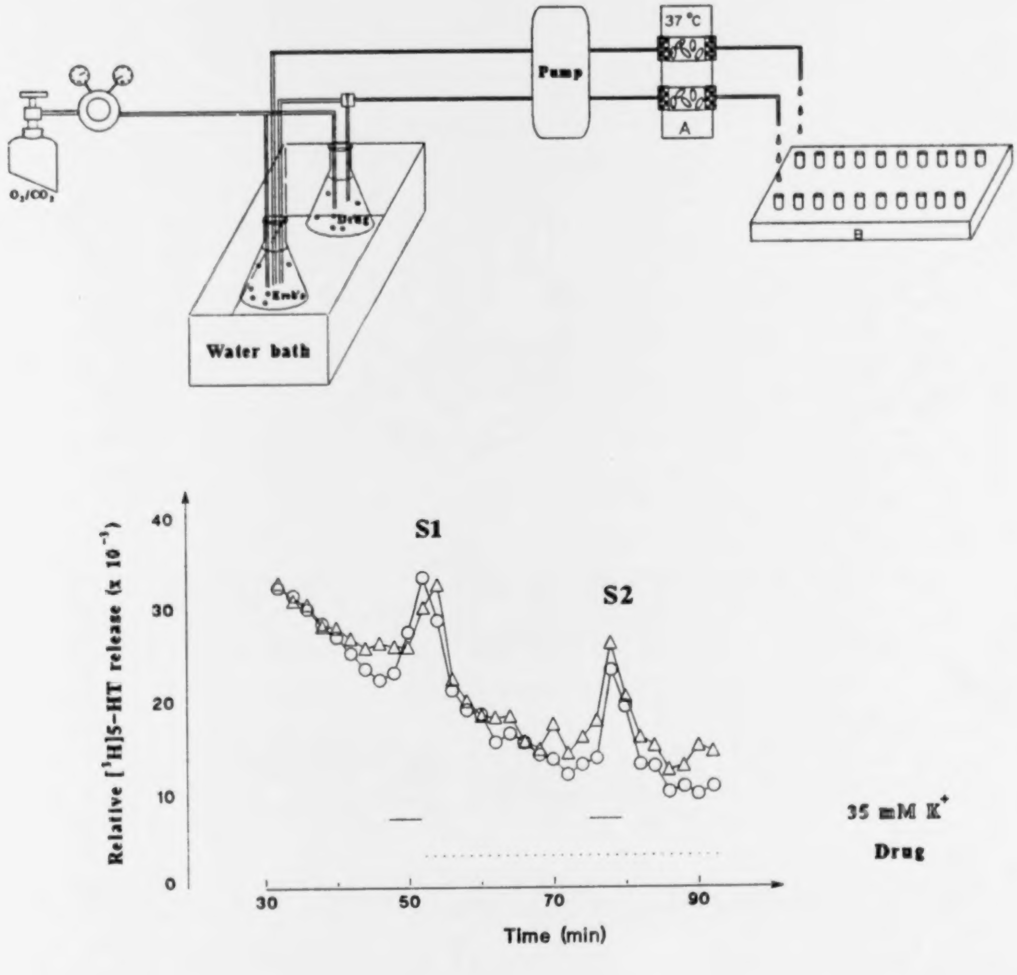

37 °C
Pump
A
B
O₂/CO₂
Krebs
Drug
Water bath
S1
S2
Relative [³H]5-HT release (x 10⁻¹)
40
30
20
10
0
30
50
70
90
Time (min)
35 mM K⁺
Drug

Figure II.1 Schematic diagram of the brain slices perifusion. A is the perifusion chamber with temperature held at 37⁰C. B is the automatic fraction collector. S_1 and S_2 represent the release of 5-HT stimulated by 35 mM K^+ kreb's medium.

Therefore, *in vivo* microdialysis was used to investigate the effect of opioids on 5-HT release from the septum of ground squirrels.

Construction of microdialysis probes: The microdialysis probes were constructed using hollow dialysis fibre (molecular weight cut off 6000 Dalton, OD. 250 μm, Spectrum), fused silica capillary tubing (World Precision Instruments Inc.), 23-gauge thin-wall stainless steel tubing (Small Parts Inc.), and PE-50 tubing (Clay Adams) as shown in Figure II.2. Briefly, one end of a piece of 23-gauge stainless steel tubing (about 18 mm in length) was inserted into a piece of PE-50 tubing (10 mm) to about 3 mm. A piece of fused silica capillary tubing (about 25 mm in length) was inserted into the steel tubing with one end extruding by 1.9 mm. With the aid of a 31-gauge hypodermic needle, the other end of the fused silica tubing pierced through the PE-50 tubing near its joint with the 23-gauge stainless-steel tubing. A small piece of blue tip (Fisherbrand pipet tips for pipetman, 1000 μl) was placed around the joint area of the PE-50 and the fused silica tubing filled with epoxy glue to strengthen the joint. A piece of hollow dialysis fibre (10 mm in length) was sealed at one end using epoxy glue and air dried overnight. The microdialysis fiber was glided over the fused silica tubing, leaving about 0.1 mm space between the sealed end of the dialysis fibre and the tip of the silica tubing and the remaining part of the dialysis fibre was inserted into the stainless steel tubing and affixed to it using epoxy glue.

Cannulation: Both non-hibernating and hibernating (in inter-bout euthermia) Columbian ground squirrels were anaesthetized with halothane and cannulated unilaterally with a 19-gauge stainless steel guide cannula in the lateral septum. Since the base of the brain of the ground squirrel is tilted at an angle of 35°, it was necessary to re-adjust the horizontal zero plane. This was achieved by moving the incisor bar downward to a distance equal to the distance between the interaural line (earbar) and the incisor bar times Sin35°. The tip of the guide

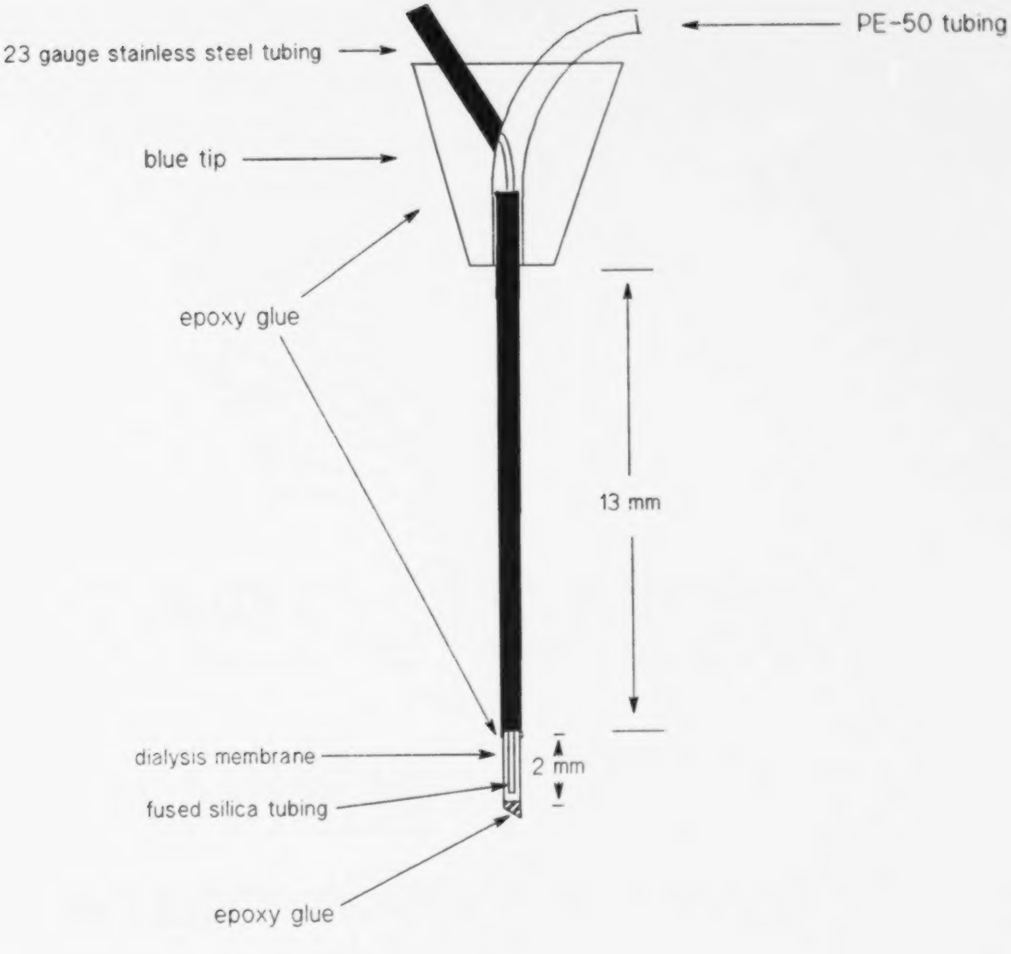

23 gauge stainless steel tubing
PE-50 tubing
blue tip
epoxy glue
13 mm
dialysis membrane
2 mm
fused silica tubing
epoxy glue

Figure II.2 Schematic illustration of constructing a microdialysis probe.

cannula was positioned 2 mm above the intended perfusion region with the coordinate of AP=13 X CR mm, L=0.7 X CR mm, H=3 X CR mm, where CR (correction factor) was the distance between the interaural line and the incisor bar divided by 36.8 mm (average distance of all animals used for preparing the atlas). The guide cannula was fixed on the skull of the animal by dental cement and sealed with a 23-gauge stylet.

Sampling and analysis microdialysis samples: On the day of experiment, non-hibernating squirrels were placed in the lab for at least one hour before the experiment, allowing them to accustomize to the environment. Ground squirrels in hibernation phase used in the experiment were warmed up at room temperature (22°C) at least six hours prior to experiment on the third day of the hibernation bout. They were used only after completely aroused (Tb=37°C). A microdialysis probe with a tip of 2 mm was then inserted into the guide cannula in the lateral septum. The squirrel was then perfused with artificial CSF (NaCl 128 mM, KCl 2.55 mM, $CaCl_2$ 1.26 mM, $MgCl_2$ 0.94 mM) containing 0.005% sodium metabisulfate at 2 μl/min using a CMA/100 microinjection pump (Carnegie Medicine). Different opioid agonists at various concentrations were added in the CSF after 60 minutes of perfusion. The animal was not handled throughout the experimental period and its activity was monitored at all times. The samples were collected every 15 minutes and immediately frozen in dry ice. The amount of 5-HT and its major metabolite 5-HIAA in the perfusate were analysed by reverse-phase HPLC using a C_{18} column (CSC-Spherisorb-ODS2, 5 μm, 15 x 0.46 cm) coupled with an electrochemical detector (ESA Coulochem model 5100A). The composition of the mobile phase was 9% MeOH in 0.09 M CH_3OONa, 0.035 M citric acid, 0.13 mM EDTA, and 0.46 mM 1-octanesulfonic acid sodium salt (pH 4.0). The flow rate was set at 0.8 ml/min. The potential setting of the conditioning cell was -0.3 V and those of the dual analytical detector were 0.05 V and 0.25 V for cell 1 and 2, respectively. The precise site of cannulation was determined histologically after completion of the experiments.

Statistics The effects of different opioid agonists on 5-HT release and the impact of these ligands on ground squirrels under different hibernation states were examined using unpaired t-test or the analysis of variance (ANOVA, one-way). The differences in modulatory effects of opioids on 5-HT release in difference hibernating states were analysed using two-way ANOVA. Significance was set at $P < 0.05$. Results are expressed as mean ± standard error.

ChemicalsDAGO, DADLE, D-Ala^2-met-enkephalinamide naloxone hydrochloride, tetrodotoxin (TTX), pargyline hydrochloride, and ascorbic acid were purchased from Sigma. U50488 and nor-BNI (nor-binaltorphimine dihydrochloride) were obtained from Research Biochemicals Incorporated. [^{3}H]5-HT creatinine sulfate (14.5 Ci/m mol) was an Amersham product. Other chemicals were obtained from BDH.

Results

Effect of three types of opioid agonists on K^+-stimulated [^{3}H]5-HT release from the hippocampal slices of Richardson's ground squirrels in non-hibernating and hibernating states

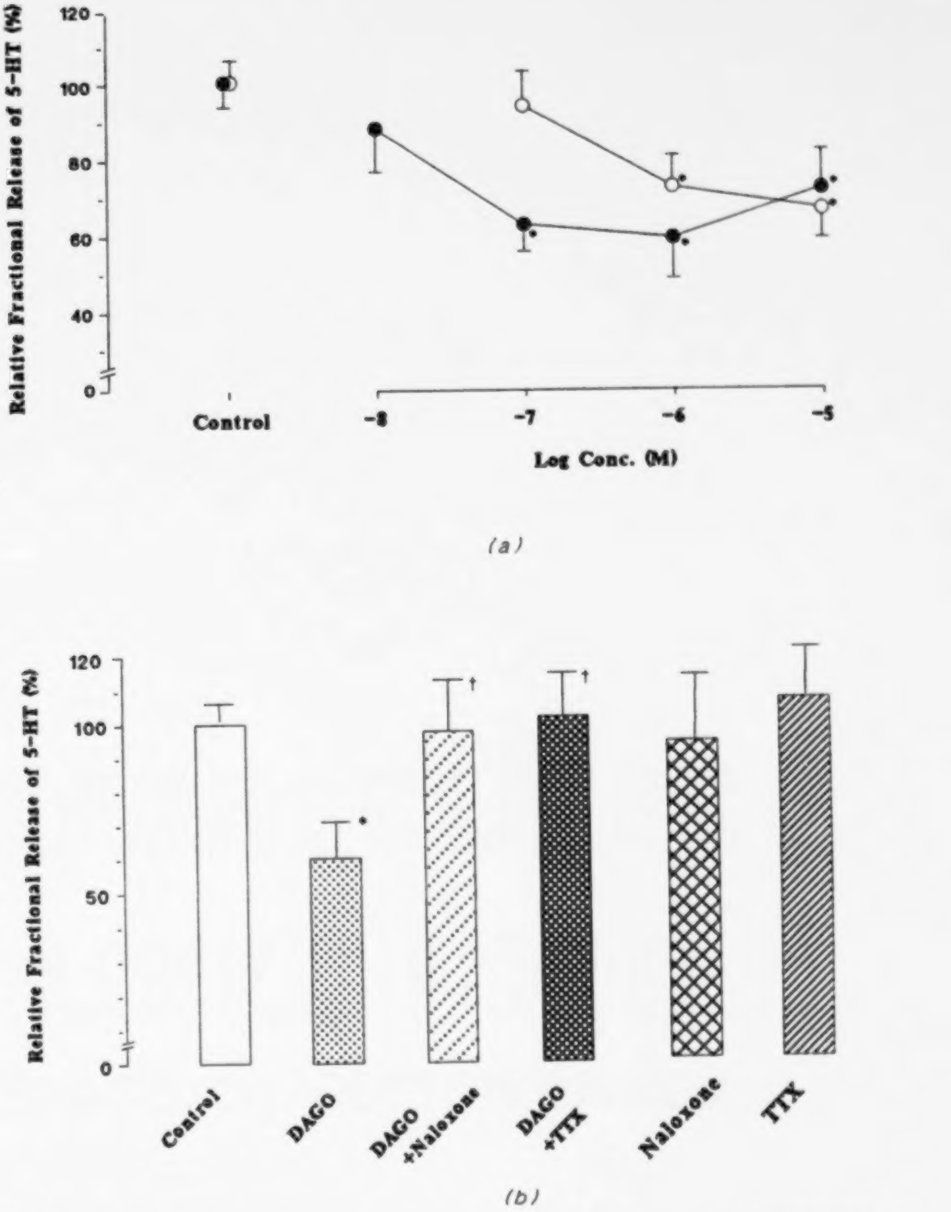
Effect of DAGO

The [^{3}H]5-HT outflow elicited by 35 mM K^+ in the hippocampal slices of the non-hibernating ground squirrels during the first period of stimulation (S_1) was $8.70 \pm 0.42\%$ of the total radioactivity and the ratio of S_2/S_1 was 0.71 ± 0.04 in the control group (n=8). The inclusion of DAGO, a μ agonist, did not alter the baseline value of [^{3}H]5-HT release. However, the addition of DAGO at the concentrations of 10^{-8} - 10^{-5} M caused a dose-dependent ($F_{4,30}=4.61$, $P<0.01$, one-way ANOVA) inhibition of 35 mM K^+-evoked [^{3}H]5-HT release from the hippocampal slices. The inhibition was significant when 10^{-7} to 10^{-5} M of DAGO was used (Figure II.3a). The maximal inhibition of 40% on [^{3}H]5-HT release was achieved at 10^{-6} M DAGO. The inhibitory effect of DAGO on K^+-stimulated [^{3}H]5-HT release was significantly diminished by both opioid antagonist naloxone (10^{-6} M) and the voltage-dependent sodium channel blocker TTX (10^{-6} M) (Figure II.3b). However, neither naloxone nor TTX alone had any significant effect on the K^+-evoked [^{3}H]5-HT release (91.08±8.27% and 102.52±9.08% of the control value for naloxone and TTX respectively, n=6).

In hippocampal slices from the hibernating ground squirrels, the [^{3}H]5-HT efflux from the control slices during the first stimulation (S_1) was 9.12±0.82% and S_2/S_1 was 0.68±0.04 (n=8). These were not significantly different from those of non-hibernating squirrels. DAGO, though less effective, still inhibited [^{3}H]5-HT release from the hippocampus of hibernating squirrels in a dose-related pattern ($F_{3,33}=4.29$, $P=0.012$, one-way ANOVA) at concentrations of 10^{-7}-10^{-5} M. A ten-fold higher concentration of DAGO than used in the non-hibernating squirrels was required to elicit a significant inhibition (Figure II.3a). The inhibition of [^{3}H]5-HT release at 10^{-5} M was about 35%. However, the right-hand shifted dose-response curve in hibernating state was not significantly different from that of non-hibernating squirrels at the doses of 10^{-7} to 10^{-5} M (the main effect of states $F_{1,63}=2.01$, $P=0.16$, two-way ANOVA).

Effect of DADLE

Similar to the μ agonist, DADLE, a δ agonist, also inhibited K^+-stimulated [^{3}H]5-HT release from the hippocampal slices of the non-hibernating ground squirrels in a dose-related pattern ($F_{3,24}=4.29$, $P=0.02$, one-way ANOVA) (Figure II.4a). Administration of DADLE did not cause a shift of basal [^{3}H]5-HT outflow. Unlike DAGO, DADLE only significantly inhibited K^+-stimulated [^{3}H]5-HT release at 10^{-5} M and the efficacy of its inhibition was also lower (27%) than that



(a)

(b)

Figure II.3 Modulatory effect of the μ agonist DAGO on K^+-stimulated $[^3H]$5-HT release from the hippocampal slices. (a) Dose-response curve of DAGO on $[^3$H5-HT release from the non-hibernating (●) and hibernating (○) Richardson's ground squirrels. (b) Antagonism of naloxone (10^{-6} M) and TTX (10^{-6} M) on the inhibitory effect of DAGO (10^{-5} M) on $[^3H]$5-HT release from the hippocampal slices of non-hibernating animals. * means significantly different from the control ($P<0.05$), whereas † significantly different from DAGO alone ($P<0.05$).

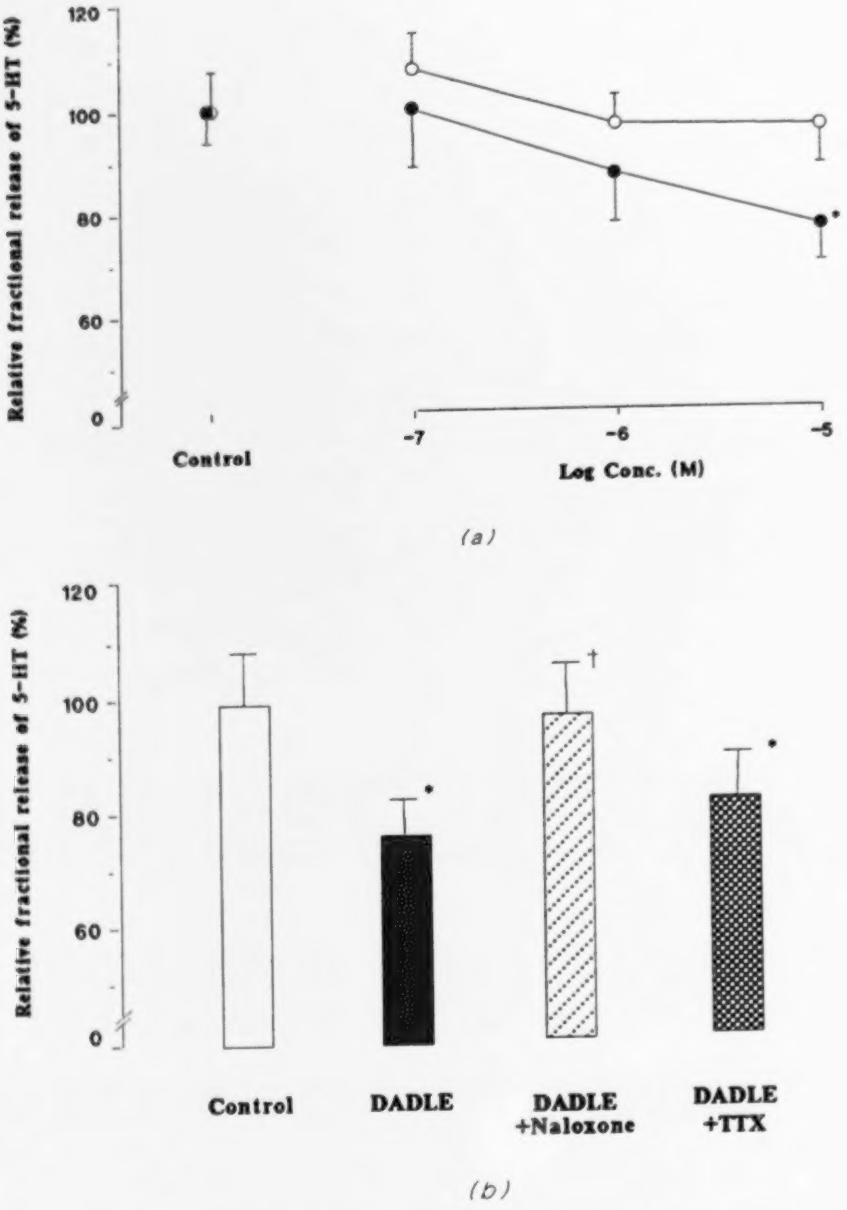


Figure II.4 Inhibitory effect of the δ agonist DADLE on K⁺-stimulated [^{3}H]5-HT release from the hippocampal slices. (a) Dose-response curve of DADLE on [^{3}H]5-HT release from the non-hibernating (●) and hibernating (○) Richardson's ground squirrels. (b) Antagonism of naloxone (10^{-5} M) and TTX (10^{-6} M) on the inhibitory effect of DADLE (10^{-5} M) on [^{3}H]5-HT release from the hippocampal slices. *: significantly different from the control ($P<0.05$); †: significantly different from DADLE alone ($P<0.05$).

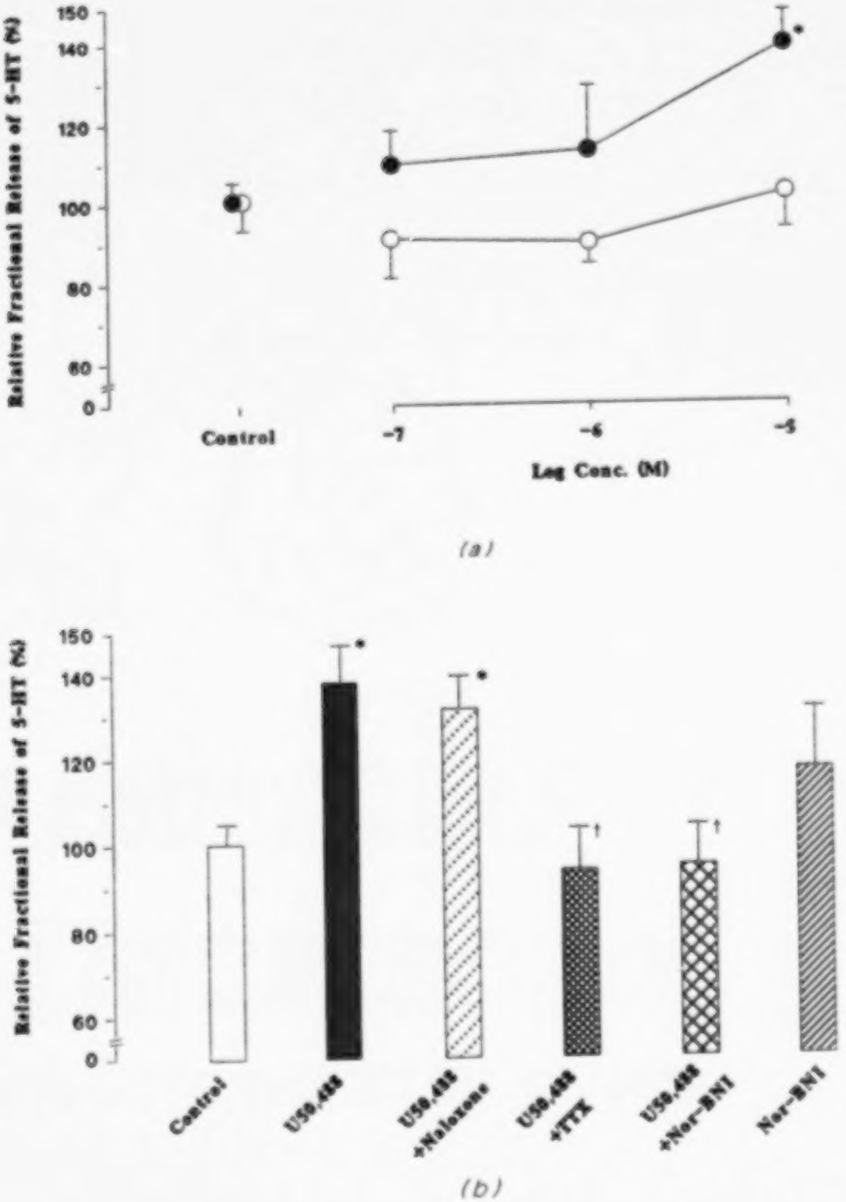
of DAGO (40%). This inhibition of $[^3H]$5-HT release could be attenuated by simultaneous inclusion of the opioid antagonist naloxone (10^{-5} M) (Figure II.4b), but TTX (10^{-6} M) had no effect on the inhibitory effect of DADLE on $[^3H]$5-HT release (Figure II.4b).

During hibernation, the inhibitory role of opioid agonist DADLE on K^+-stimulated $[^3H]$5-HT release was diminished ($F_{3,16}=1.75$, $P=0.20$, one-way ANOVA) (Figure II.4a). The change in pattern of dose-response curves of DADLE inhibiting $[^3H]$5-HT release from the hippocampus of non-hibernation and hibernating ground squirrels was state dependent ($F_{1,40}=5.87$, $P=0.02$, two-way ANOVA).

Effect of U50488

In contrast to what was observed with DAGO and DADLE, U50488, a κ agonist, induced a dose-dependent ($F_{3,38}=5.27$, $P<0.01$, one-way ANOVA) enhancement of K^+-stimulated $[^3H]$5-HT release from the hippocampal slices of non-hibernating ground squirrels (Figure II.5a). It elevated $[^3H]$5-HT release by 37% at the concentration of 10^{-5} M. Inclusion of U50488 in the perifusion medium did not alter the basal $[^3H]$5-HT value. The stimulatory effect of U50488 could not be blocked by naloxone (10^{-5} M), but was completely abolished by either nor-BNI (10^{-6} M), a specific κ antagonist, or TTX (10^{-6} M) (Figure II.5b). Neither nor-BNI nor TTX alone altered the release of $[^3H]$5-HT (96.05 ± 8.43% and 102.52 ± 9.08%, respectively).

In contrast to the non-hibernating squirrels, the stimulatory effect of U50488 on K^+-stimulated $[^3H]$5-HT release was completely absent in the hibernating squirrels ($F_{3,27}=0.69$, $P=0.57$, one-way ANOVA) (Figure II.5a). This change in dose-response curves of κ agonist U50488 on $[^3H]$5-HT release was significantly state-dependent (the main effect of state was $F_{1,65}=9.11$, $P<0.01$, two-way



(a)

(b)

Figure II.5 Stimulatory effect of the κ agonist U50488 on K$^+$-stimulated [^{3}H]5-HT release from the hippocampal slices. (a) Dose-response curve of U50488 on [^{3}H]5-HT release from the non-hibernating (●) and hibernating (○) Richardson's ground squirrels. (b) Antagonism of naloxone (10^{-5} M), nor-BNI (10^{-6} M), and TTX (10^{-6} M) on the stimulatory effect of U50488 (10^{-5} M) on [^{3}H]5-HT release from the hippocampal slices. *: significantly different from the control ($P<0.05$); †: significantly different from U50488 alone ($P<0.05$).

ANOVA).

Effects of DAGO, DADLE, and U50488 on K$^+$-stimulated [^{3}H]5-HT release from the hypothalamic slices of the Richardson's ground squirrels under non-hibernating and hibernating states

As the hypothalamus is one of the few regions which exhibits neuronal electrical and metabolic activities during hibernation, the modulatory effect of DAGO, DADLE, and U50488 on K$^+$-stimulated [^{3}H]5-HT release was also examined in the hypothalamic slices. The amount of [^{3}H]5-HT released in the control of non-hibernating squirrels (S_1=9.34±0.67%, S_2/S_1=0.63±0.04, n=6) was similar to that observed in the hibernating animals (S_1=9.54±0.65%, S_2/S_1=0.65±0.03, n=6). Different from the observations in the hippocampal slices, none of DAGO, DADLE, and U50488 had any significant effects on K$^+$-stimulated [^{3}H]5-HT release from the hypothalamic slices of either non-hibernating or hibernating ground squirrels (Table II.1). In addition, none of the three opioid agonists altered the basal [^{3}H]5-HT outflow from the hypothalamic slices.

Effects of DAGO, DADLE, and U50488 on K$^+$-stimulated [^{3}H]5-HT release from the hippocampal slices of non-hibernating Columbian ground squirrels

To elucidate whether there is a species difference in the modulatory effects of various opioid agonists on [^{3}H]5-HT release, hippocampal slices of six non-hibernating Columbian ground squirrels were also used in the brain slice perifusion study. The [^{3}H]5-HT outflow from the first K$^+$-stimulation (S_1) was 12.12 ± 0.83% (n=6) of the total [^{3}H]5-HT content of the slices which was significantly higher than that of the Richardson's ground squirrel (P=0.004). However, the S_2/S_1 ratio was 0.73 ± 0.03 (n=6) in control slices of Columbian ground squirrels which was almost the same as that of the Richardson's ground squirrels. The μ agonist DAGO at a concentration of 10^{-5} M also significantly inhibited K$^+$-stimulated [^{3}H]5-HT release by 26% (P=0.03, t-test) (Table II.2).

TABLE II.1

The modulatory effects of opioid agonists on 5-HT release from hypothalamic slices of non-hibernating and hibernating Richardson's ground squirrels[†]

	Non-hibernating	Hibernating
Mu agonist DAGO		
Control	100.00 ± 7.03%	100.00 ± 4.94%
10^{-7} M	89.87 ± 3.57%	103.02 ±11.87%
10^{-6} M	96.68 ± 4.71%	99.37 ± 7.67%
10^{-5} M	84.72 ± 6.33%	103.30 ±11.63%
Delta agonist DADLE		
Control	100.00 ± 4.26%	100.00 ± 3.16%
10^{-7} M	120.95 ± 6.39%	113.21 ± 7.31%
10^{-6} M	104.92 ± 2.64%	100.33 ± 3.76%
10^{-5} M	94.00 ± 5.38%	106.79 ±11.09%
Kappa agonist U50488		
Control	100.00 ± 5.29%	100.00 ± 4.91%
10^{-7} M	94.34 ±15.58%	87.87 ±13.12%
10^{-6} M	95.23 ± 9.30%	109.39 ± 7.40%
10^{-5} M	96.08 ±11.40%	105.35 ± 8.13%

†: The results are mean ± s.e.m. of five to seven experiments using control as 100%.

TABLE II.2 The modulatory effects of opioid agonists on 5-HT release from hippocampal slices of non-hibernating Columbian ground squirrels

	Non-hibernating	T-test P value
Control	100.00 ± 3.92%	
Mu agonist DAGO, 10^{-5} M	74.06 ± 9.39%	0.029
Mu agonist DAGO, 10^{-6} M	90.90 ± 6.34%	0.250
Delta agonist DADLE, 10^{-5} M	82.31 ± 5.69%	0.028
Kappa agonist U50488, 10^{-5} M	111.22 ± 7.97%	0.235

The results are mean ± s.e.m. of six animals using control as 100%.

The inhibitory effects of δ agonist DADLE (10^{-5} M) on K^+-stimulated [^{3}H]5-HT release (18%) was similarly significant. Although insignificant, κ agonist U50488 stimulated [^{3}H]5-HT release by 11% (Table II.2). Therefore, the general pattern of various opioid agonists on modulating K^+-stimulated [^{3}H]5-HT release from the hippocampal slices of both Richardson's and Columbian ground squirrels appears to be similar.

In vivo study on the regulatory role of various opioids on 5-HT apparent turnover in the lateral septum of Columbian ground squirrels via microdialysis

From the brain slices perifusion study on the modulatory role of opioids on K^+-stimulated [^{3}H]5-HT release, species difference between Richardson's and Columbian ground squirrels did not seem to be profound. In addition, because of the more docile nature of the Columbian ground squirrels, this species was used in microdialysis studies instead of the more irritable Richardson's ground squirrels.

The squirrels were perfused with artificial CSF in the lateral septum at 2 μl/min for 60 minutes before the medium was switched to CSF containing various opioid agonists. After 45 minutes of perfusion with the opioid agonist, the dialysis medium was changed back to CSF for another 30 minutes. Most of the animals used in the experiments were quiet and resting during the dialysis period. The amount of 5-HT and 5-HIAA in the dialysate was detected with HPLC-EC. The retention times for 5-HIAA and 5-HT were 17 minutes and 28 minutes, respectively (Figure II.6). The detection limit of the system (signal/noise ratio of 2) was 8 pg for 5-HT and 5 pg for 5-HIAA.

The basal 5-HT and 5-HIAA apparent turnover in the lateral septum was calculated by averaging their turnover before the application of opioid agonist (from minute 30 to minute 60 after the perfusion started). The effect of opioids

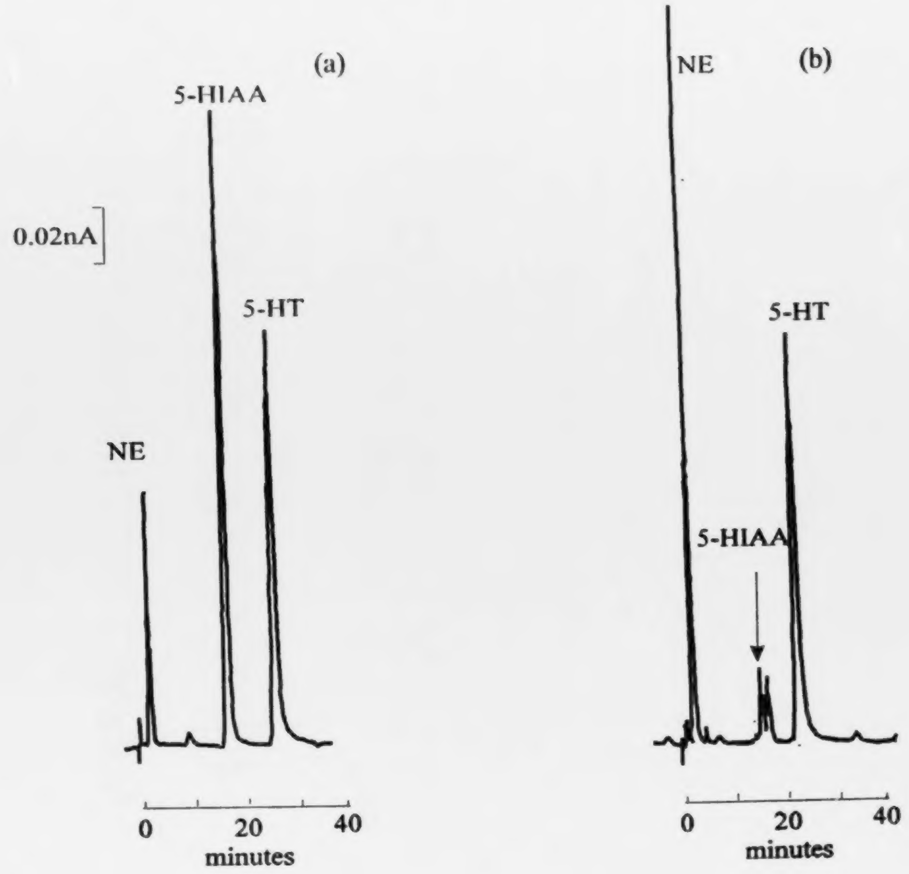


Figure II.6 HPLC-EC detection of 5-HT and 5-HIAA in microdialysis sample obtained from the lateral septum of the Columbian ground squirrels. (a) standards: 400 pg of NE, 5-HIAA, and 5-HT; (b) dialysate sample.

on apparent turnover of 5-HT and 5-HIAA was expressed as percentage of the basal 5-HT or 5-HIAA turnover calculated as above mentioned. The average basal 5-HT apparent turnover obtained from the septum of six Columbian ground squirrels in their non-hibernation phase was 0.051 ± 0.008 and 5-HIAA was 0.042 ± 0.013 pmol/ min. The 5-HT basal apparent turnover was not significantly affected by application of 0.1 mM of DAGO in the dialysis medium (Table II.3, Figure II.7), but 5-HIAA turnover was significantly suppressed by 0.1 mM DAGO ($F_{1,30}$=4.05, P=0.05). The κ agonist U50488 (at 0.65 mM) did not alter the 5-HT apparent turnover nor that of the 5-HIAA (Table II.3, Figure II.8). However, administration of 0.17 mM D-Ala^{2}-met-enkephalinamide, a δ agonist, significantly elevated 5-HT and 5-HIAA apparent turnover by over 200% and 50%, respectively (Table II.3, Figure II.9). In the septum of artificially aroused hibernating Columbian ground squirrels, the basal 5-HT apparent turnover was 0.031 ± 0.005 pmol/min (n=4) which was significantly lower than that of non-hibernating animals ($F_{1,29}$=6.38, P=0.02, one-way ANOVA). Similarly, the 5-HIAA turnover was also lower in squirrels in their hibernating phase (0.016 ± 0.003 pmol/min). Similarly to non-hibernating phase, neither DAGO nor U50488 had any significant impact on 5-HT and 5-HIAA apparent turnover in the hibernating phase (Table II.3, Figure II.7, 8). However, D-Ala^{2}-met-enkephalinamide, in contrast to that observed in non-hibernating animals, had no significant impact on 5-HT and 5-HIAA apparent turnover in the hibernating phase (Table II.3, Figure II.9).

Table II.3 The influence of three types of opioid agonist on apparent turnover of 5-HT and 5-HIAA in the septum of non-hibernating and hibernating Columbian ground squirrels

	5-HT	ANOVA P value	5-HIAA	ANOVA P value
Non-hibernating phase (n=6)				
Basal apparent turnover (pmol/min)	0.051 ± 0.008 (as 100%)		0.042 ± 0.013 (as 100%)	
Max. Response after application of				
mu agonist DAGO				
0.01 mM	119.69%	N/A	108.07%	N/A
0.10 mM	140.29%	0.37	68.91%	0.05
delta agonist D-Ala^2-met-enkephalinamide				
0.09 mM	197.76%	N/A	100.11%	N/A
0.17 mM	351.42%	0.02	151.66%	0.01
kappa agonist U50488				
0.32 mM	153.06%	N/A	113.60%	N/A
0.65 mM	220.66%	0.11	83.41%	0.88
Hibernating phase (n=4)				
Basal apparent turnover (pmol/min)	0.031 ± 0.005 (as 100%)†		0.016 ± 0.003 (as 100%)†	
Max. Response after application of				
mu agonist DAGO				
0.10 mM	62.84%	0.25	71.56%	0.17
delta agonist D-Ala^2-met-enkephalinamde				
0.17 mM	55.34%	0.21	156.65%	0.21
kappa agonist U50488				
0.65 mM	160.58%	0.25	171.09%	0.92

The values in the table are the average from six non-hibernating or four hibernating Columbian ground squirrels except the lower concentration treatment which were obtained from two non-hibernating animals. N/A: not available because of small n size. †: Significantly different from the corresponding apparent turnover in the non-hibernating phase.

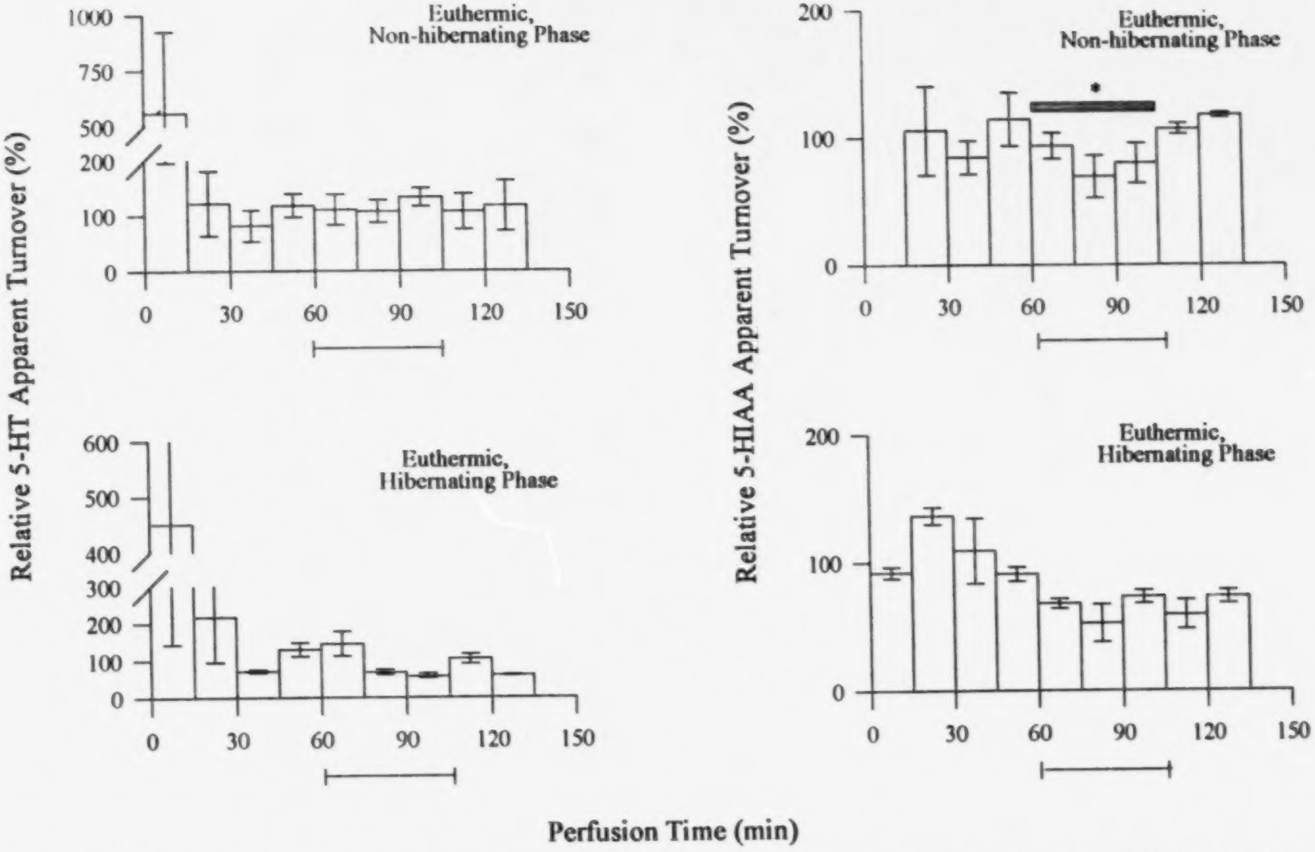


Figure II.7 The effect of the μ agonist DAGO (0.1 mM) on the apparent turnover of 5-HT and 5-HIAA in the lateral septum of the Columbian ground squirrel in the non-hibernating (n=6) and hibernating (n=4) phases. (The lower bar indicates inclusion of DAGO in the medium from minute 60 to minute 105.) Hatched bar and *: significantly differnent from basal apparent turnover (minute 30 to minute 60, $P<0.05$).

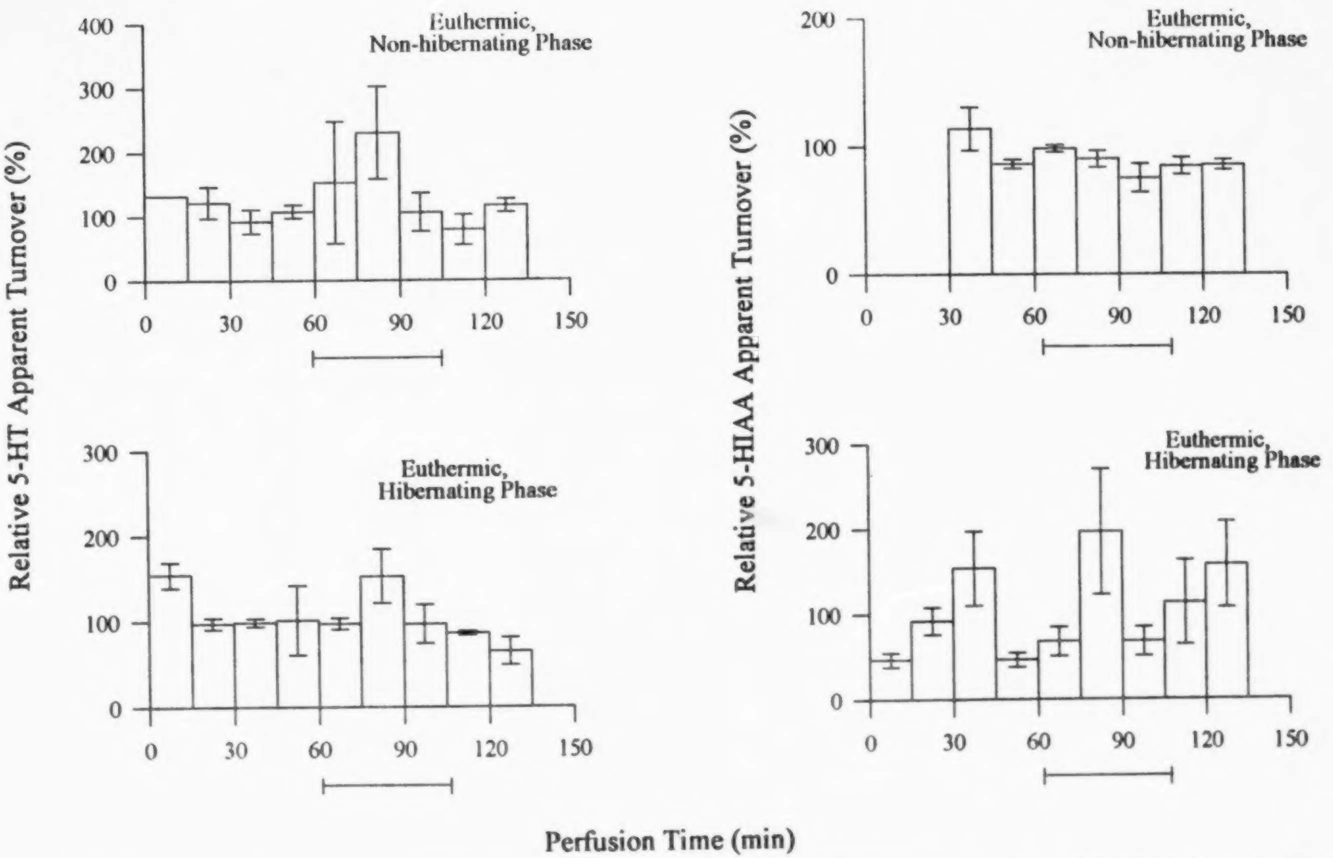


Figure II.8 The influence of the κ agonist U50488 (0.65 mM) on the apparent turnover of 5-HT and 5-HIAA in the lateral septum of the Columbian ground squirrel in the non-hibernating (n=6) and hibernating (n=4) phases. (The bar indicates inclusion of U50488 in the medium from minute 60 to minute 105.)

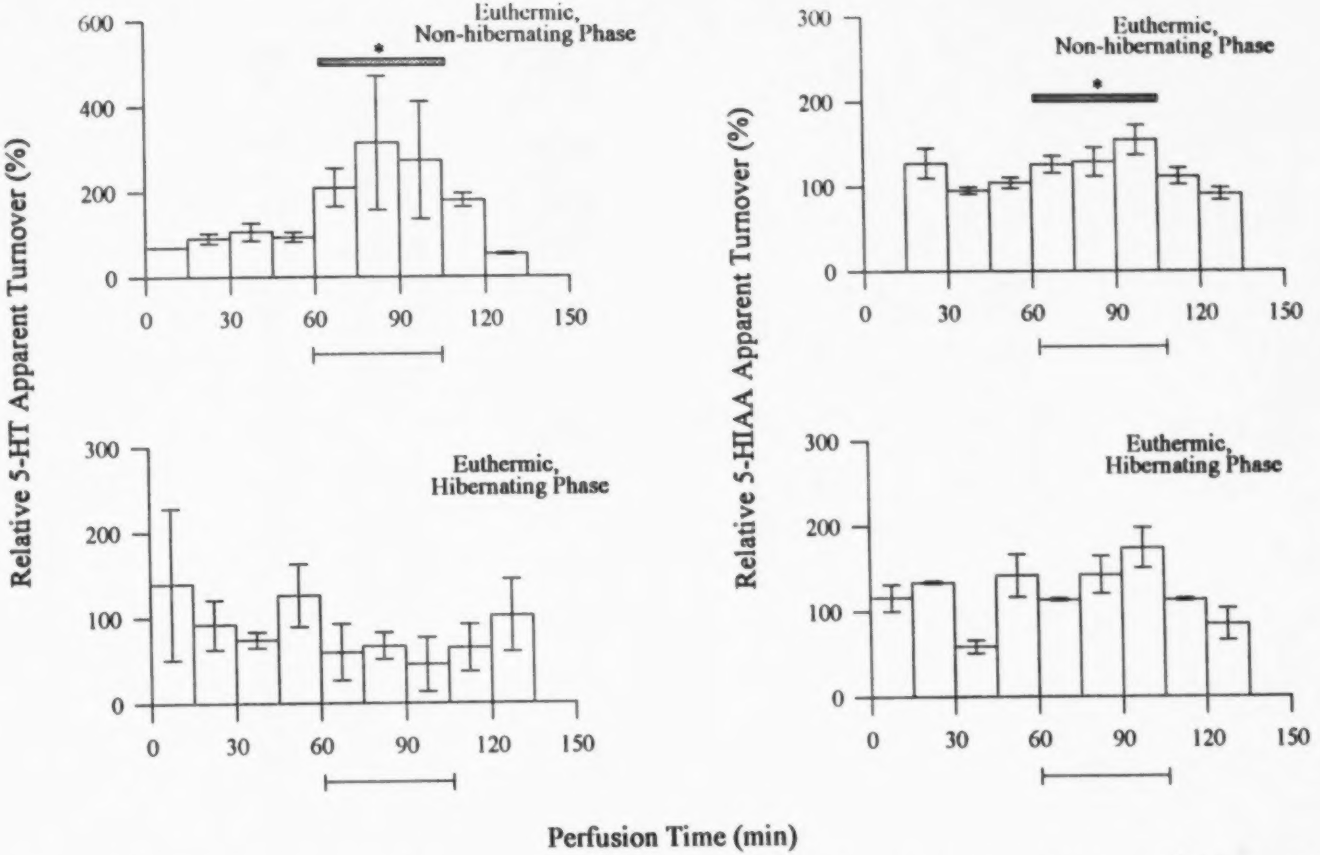


Figure II.9 The effect of the δ agonist D-Ala^2-met-enkephalinamide (0.17 mM) on the apparent turnover of 5-HT and 5-HIAA in the lateral septum of the Columbian ground squirrel in the non-hibernating (n=6) and hibernating (n=4) phases. (D-Ala^2-met-enkephalinamide, the lower bar, was added to the medium from minute 60 to minute 105.) Hatched bar and *: significantly differnent from basal apparent turnover (minute 30 to minute 60, $P<0.05$).

Discussion

To study the modulatory role of opioids on serotonergic activity in different hibernation states, two different approaches were employed. Brain slice perifusion was a rather classical and well-established assay (Lipton 1985), especially for studying modulation of neurotransmitter release. It has been demonstrated that the modulatory effect of opioid agonists on the release of ACh from rat cortical slices investigated via *in vitro* perifusion study is similar to that illustrated by *in vivo* examination (Jhamandas et al. 1975). In addition, the study of alterating adenosine receptor activity in modulating ACh release by *in vitro* perifusion (Jin et al. 1993) is in concordance with the physiological and pharmacological changes revealed by other methods (Corradetti et al. 1984). Therefore, the "reduced preparation" (Lipton 1985), i.e. brain slice perifusion, was used for larger regions, such as the hippocampus and hypothalamus. However, the large amounts of tissue required for slice perifusion is seldom met by other smaller brain regions, e.g. the septum. With the development of microdialysis technique (Benveniste 1989, Ungerstedt 1991), it is possible to monitor various neuronal activities *in vivo* with minimal damage. With microdialysis, the small and slow changes in extracellular concentration of certain neurotransmitters can be monitored during different behavioral profiles or in response to certain pharmacological manipulations (Westerink and Justice 1991) in free-moving animals. Therefore, it is a very powerful tool in studying the regulatory role of opioids in the outflow of other neurotransmitters.

The present study not only demonstrated that opioids modulate serotonergic activity in the CNS of ground squirrels similar to previous observations in other species (Passarelli and Costa 1988; Yoshioka et al. 1993), but also indicated that the modulatory effects of opioids in the ground squirrels varies with the specificity of the agonists, the actual location of the CNS regions, and the state of the animals. Although it has been indicated that acute administration of morphine increased cerebral 5-HIAA content (Ahtee and Attila 1980) and microinjection of (D-Ala^2)-methionine enkephalin into the lateral ventricle or the median raphe increased 5-HT turnover in the limbic forebrain (Algeri et al. 1978) and the hippocampus (Forchetti et al. 1982), our hippocampal slice perifusion did not evoke the same changes. This may be due to the addition of a MAO inhibitor, pargyline, and an anti-oxidant ascorbic acid, in our perifusion medium which may have prevented or minimized the hydrolysis of 5-HT to 5-HIAA. The effectiveness of the treatment was verified by the HPLC examination of the slice superfusate that over 90% of the radioactivity was in the form of 5-HT (i.e., the [^{3}H]-5-HT had not been oxidized). In microdialysis study, however, because of the less protection for 5-HT (only an anti-oxidant, sodium metabisulfate, was added to the perfusion medium), the ratio of 5-HT/5-HIAA in the perfusate collected from the lateral septum (2.5 to 4) was different from that of the slice perifusion (around 9). However, as indicated in Table II.3, the 5-HT and 5-HIAA apparent turnover was altered to different degrees by the same opioid agonist D-Ala^2-met-enkephalinamide. This agreed with previous findings that extracellular 5-HIAA does not change in concordance with the change in extracellular 5-HT concentration or serotonergic activity (Kalén et al. 1988, Sharp et al. 1989, Matos et al. 1990). This was true for metabolites of other monoamine neurotransmitters. The general lack of relationship between extracellular concentration of monoamine transmitters and their metabolites could be because the metabolites are mainly generated from the oxidation of intracellular unreleased monoamines (Grahame-Simth 1971, Commissiong 1985, Crespi et al. 1990). Therefore, the amount of metabolites in the extracellular space is considered a poor indicator of monoaminergic activity (Crespi et al. 1990, Westerink and Justice 1991).

It has been suggested (Wood 1986, Jackisch et al. 1988, Mulder et al. 1990) that opioids regulate different physiological processes by modulating other neurotransmitters through activation of specific opioid receptors. Our results showed that different opioid ligands exhibited different modulatory roles on the release of 5-HT from the hippocampal region of non-hibernating ground squirrels. Both the μ agonist DAGO and the δ agonist DADLE suppressed K^+-evoked 5-HT release from the hippocampal slices of non-hibernating Richardson's ground squirrel, whereas the κ agonist U50488 enhanced it. The stimulatory effect of κ agonists appears to be a specific activation of the κ receptors, as the response was only attenuated by the specific κ antagonist nor-BNI, but not the non-specific opioid antagonist naloxone, even at a concentration of 10 μM. Therefore, the difference in opioid-mediated 5-HT release observed in the present study may be due to the activation of different opioid receptor types in specific neuronal circuits in the hippocampus. This seems to be the case as (1) the μ agonist (optimal concentration was 10^{-6} M with 40% inhibition) appears to be more potent than the δ agonist (27% inhibition at 10^{-5} M) in suppressing 5-HT release; (2) inclusion of TTX in the perifusion medium attenuated the inhibitory effect of μ agonists, but not that elicited by δ agonists. Therefore, δ agonists may inhibit 5-HT release by acting directly on serotonergic terminals, whereas μ agonists appear to elicit their effect indirectly via another interneuron. Further, the κ agonist U50488 apparently also elicited its effect indirectly via an interneuron because the simultaneous addition of TTX in the medium abolished the response. Similar diverse effects of κ agonist versus μ and δ agonists in modulating hippocampal neuronal activity (Bradley and Brooks 1984), dopamine release (Di Chiara and Imperato 1988), and impairment of spontaneous alteration performance (Itoh et al. 1994) have also been reported. These differences may be a consequence of different mechanisms by stimulating the different opioid receptor types located in different synaptic circuits coupled to different second messenger systems (Simonds 1988). It has been shown that the μ and δ receptors may be homologous and co-exist in the same neuron as separate receptors or even associated in a complex form (Wollemann 1990, Childers 1991, Traynor and Elliott 1993). Further, the second messenger systems coupled to μ and δ receptors are also similar; both have been shown to inhibit adenylate cyclase activity and/or activate potassium channels (North et al. 1987). The κ receptor is distinctly different from the μ and δ receptors both in its molecular make-up and in its coupling to the calcium channels (Ueda et al. 1989, Wollemann 1990, Childers 1991). Therefore, it is quite possible that different types of opioid agonists affect the stimulated 5-HT release differently by activating different ion channels and second messenger pathways.

In contrast to the observed inhibitory effect of δ agonists on 5-HT release from the hippocampal slices of non-hibernating Richardson's ground squirrels, the δ agonist stimulated 5-HT outflow from the lateral septum of non-hibernating Columbian ground squirrels. This difference may not be a result of species difference since the modulatory effects of opioids on 5-HT release from hippocampal slices of Columbian ground squirrels were similar to that observed in Richardson's ground squirrels. Therefore, opioids differentially modulate 5-HT release in different CNS regions. The opposite effects of opioid in modulating hippocampal neuronal activity to that of other brain regions have also been extensively reported. For instance, met-enkephalin, β-endorphin, and normorphine inhibit the neuronal activity of opioid-responsive cells in the cerebral cortex, brainstem, caudate nucleus, and thalamus, but excite the neuronal activity of opioid-responsive cells in the hippocampus (Nicoll et al. 1977). This latter "atypical" excitation has been shown to be the result of the opioid inhibiting adjacent inhibitory interneurons (Gansberger et al. 1979), causing disinhibition (Lee et al. 1980).

Different from the effects observed in the septal and hippocampal regions, none of the three opioid agonists altered K^+-stimulated 5-HT release from the hypothalamic slices of Richardson's ground squirrels. This further demonstrated that the modulatory effects of opioids on 5-HT release is site-specific. However, the failure of regulating 5-HT release by opioids in the hypothalamus of either non-hibernating or hibernating ground squirrels is by no means suggesting that opioids do not modulate the hypothalamic neuronal activity or that opioidergic activity does not change during the hibernation cycle. Rather, it has been found that gene expression of the proenkephalin family in the periventricular hypothalamus increased significantly during hibernation (Nurnberger et al. 1994). Therefore, it is possible that an indirect modulatory pathway of opioids on 5-HT release was disturbed in the "reduced preparation" of hypothamalic slices or hypothalamic opioids may modulate the activities of neurotransmitters other than 5-HT in regulating the hibernation process.

In addition to ligand dependency and site specificity, the regulatory role of opioids on 5-HT release from the lateral septum and hippocampus of ground squirrels is also state-dependent. The inhibitory effect of DADLE in the hippocampus was only present in the non-hibernating animals. Similarly, only in the non-hibernating state did U50488 stimulate 5-HT release from the Richardsons' hippocampus and D-Ala^2-met-enkephalinamide elevate 5-HT turnover in the Columbian's lateral septum. However, in the hibernating state there is a lack of responsiveness of 5-HT efflux to DADLE, U50488, and D-Ala^2-met-enkephalinamide in both squirrels. This sensitivity change appears to be receptor specific as the suppressive effect of μ agonist on 5-HT release from the Richardsons' hippocampus persisted, though decreased slightly, in the hibernating animals. Moreover, as the pattern of K^+-stimulated 5-HT release from the hippocampal slice of control groups were about the same in both hibernating and non-hibernating animals, it is unlikely that the failure of DADLE, U50488, and D-Ala^2-met-enkephalinamide to affect the 5-HT outflow in the hibernating animals was due to a general depression of neuronal activity during hibernation. This is further strengthened by recent findings that the general CNS depression induced by anaesthesia could not prevent the development of physical dependence to morphine observed during hibernation (Beckman et al. 1993). Rather, the reduction in responses to opioids may indicate an endogenous change in the hippocampal and septal opioid system during the hibernating state. To date, there has been some evidence indicating an increased brain opioid activity during the hibernating phase (Kromarova et al. 1983; Nurnberger et al. 1991). It is quite possible that the reduced responsiveness to DADLE, U50488, and D-Ala^2-met-enkephalinamide observed in the hibernating squirrels may reflect a reduction in the responsiveness of opioid receptors consequent to an enhanced endogenous opioid activity prior to or in the early hibernation phase. This interpretation is supported by previous observation that the binding of dihydromorphine, a relative non-specific opioid ligand with μ preference, in the hippocampus decreased in the hibernating golden-mantled ground squirrel (Beckman et al. 1986). In contrast to the reduced sensitivity to δ and κ agonists, the modulatory effect of the μ agonist still remained during the hibernating state. This may be interpreted to suggest that the hippocampal μ type opioid receptors have not been down-regulated to the same extent as δ and κ receptors during hibernation. This may also be due to the differences in distribution of the three opioid receptors. However, the exact physiological significance of these variations is still unknown.

As it has been reported that different opioid agonists elicit opposing neuronal responses in the hippocampus of rats and guinea pigs (Wagner et al. 1992; Dingledine 1985), the modulatory effects of opioids on 5-HT release from the hippocampus of ground squirrels was also different from that of rats. U50488 stimulated 5-HT release from the hippocampus of non-hibernating ground squirrels, whereas, at the same concentration, it suppressed 5-HT efflux from that of rats (Cui et al. 1994). This difference in pharmacological responses may be due to species differences in distribution of the three opioid receptor types which has been shown previously (Herkenham and McLean 1988). However, the significance of species differences in relation to a receptor's physiological function and its possible adaptive value is still unknown.

In relation to the regulation of hibernation, the physiological significance of state-dependent changes in opioid-modulated 5-HT release from the septum and hippocampus remains unclear. Based on the current model of neuronal regulation of Tb in hibernation (Heller 1979; Beckman and Stanton 1982) and neural activity in the hibernating animals (Belousov 1993, Pakhotin et al. 1993), inhibition of the BSRF by the septo-hippocampal complex is a fundamental determinant in initiating and maintaining the depression of Tb and general autonomic suppression. The physiological activity of both opioids and 5-HT is primarily inhibitory (Chesselet 1984; Segal 1975); it is possible that opioids and 5-HT within the septum and hippocampus may work in concert to regulate the onset and maintenance of hibernation. Furthermore, the differences in the modulatory effect of different opioids on 5-HT release may suggest that they play different roles in the regulation of the hibernation cycle.

References

Ahtee L and Attila L.M.J. (1980) Opioid mechanism in regulation of cerebral monoamines in vivo. Histochemistry and cell biology of autonomic neurons, SIF cells, and paraneurons Eranko O., Soinila S., and Paivarinta H. (eds), pp 361-365, Raven Press, New York.

Akil H., Watson S. T., Yang E., Lewis M. E., Khaehaturian H. and Waller J. M. (1984) Endogenous opioids: biology and function. Ann Rev Neurosci 7:223-255

Akil H., Bronstein D., and Mansour A. (1988) Overview of the endogenous opioid systems: anatomical, biochemical and functional issues. Endorphins, opiates and behavioral processes Rogers R.J. and Cooper S.J. (eds), pp 1-23, Chichester, New York.

Algeri A., Consoluzione A., Calderini G., Achilli G., Puche Canas E., Garattini S. (1978) Effects of the administration of (D-Ala^2)methionine-enkephalin on the serotonin metabolism in the rat. Experientia 34:1488-1489

Angevine J.B. and Cotman C.W. (1981) Principles of neuroanatomy Oxford University Press, New York.

Azmitia E.C. and Segal M. (1978) An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat J Comp Neurol 179:641-668

Barnard E. A. and Simon J. (1993) Opioid receptors. Neurotransmitter Receptors Hucho F. (ed), pp 297-323, Elsevier, Amsterdam.

Beckman A.L. and Stanton T.L. (1982) Properties of the CNS during the state of hibernation The neural basis of behaviour Beckman A.L. (ed), pp 19-45, Spectrum, New York.

Beckman A., Dean R. R.,and Wamsley J. K. (1986) Hippocampal and cortical opioid receptor binding: changes related to the hibernation state. Brain Res 386:223-231

Beckman A.L., Beaver T.A., and Lewis F.A. (1993) Morphine physical dependence in the hibernator: cental nervous system mechanisms underlying the development of dependence remain functional during depression induced by pentobarbital anaesthesia. Life Sci. 52:1079-1086

Belousov A.B. (1993) On possible mechanisms of the septohippocampal system participation in the neural control of hibernation. Life in the cold: Ecological, physiological, and molecular mechanism Carey C., Florant G.L., Wunder B.L., and Horwitz B. (eds), pp 291-296, Westview Press, Colorado.

Benveniste H. (1989) Brain microdialysis. J Neurochem 52:1667-1679

Berger A. and Ramire A. J. (1988) The central hypotensive effect induced by methionine-enkephalin involves a serotonergic pathway. J Hypertension 6:S514-S517

Bradley P.B. and Brooks A. (1984) A microiontophoretic study of the actions of μ-, δ- and κ-opiate receptor agonists in the rat brain. Br J Pharmacol 83:763-772

Canguilhem B., Miro J.L., Kemph E., and Schmitt P. (1986) Does serotonin play a role in entrance into hibernation? Am J Physiol 22:701-708

Chesselet M.F. (1984) Presynaptic regulation of neurotransmitter release in the brain: facts and hypothesis. Neuroscience 12:347-375

Childers S.R. (1991) Opioid receptor-coupled second messenger systems. Life Sci 48:1991-2003

Cohen G.A., Doze V.A., and Madison D.V. (1992) Opioid inhibition of GABA release from presynaptic terminals of rat hippocampal interneurons. Neuron 9:325-335

Commissiong J.W. (1985) Monoamine metabolites: their relationship and lack of relationship to monoaminergic neuronal activity. Biochem Pharmacol 34:1127-1131

Corradetti R., Kiedrowski L., Nordstrom O., and Pepeu G. (1984) Disappearance of low affinity adenosine binding sites in aging rat cerebral cortex and hippocampus. Neurosci Lett 49:143-146

Crespi F., Garratt J.C., Sleight A.J., and Marsden C.A. (1990) In vivo evidence that 5-hydroxytryptamine (5-HT) neuronal firing and release are not necessarily correlated with 5-HT metabolism. Neurosci 35:139-144

Cui Y., Lee T.F., and Wang L.C.H. (1994) Species difference in the modulatory effect of *kappa* agonist on 5-HT release from ground squirrel and rat hippocampus. Neurosci Lett 175:126-128

Di Chiara G. and Imperato A. (1988) Opposite effects of *mu* and *kappa* opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. J Pharmacol Exp Ther 244:1067-1080

Dingledine R. (1985) Opioid Peptides: Central Nervous System. Neurotransmitter Actions in the Vertebrate Nervous System Rogawski M.A. and Barker J.L. (eds), pp 364-431, Plenum Press, New York.

Forchetti C.M., Marco E.J., and Meek J.L. (1982) Serotonin and γ-aminobutyric acid turnover after injection into the median raphe of substance P and D-Ala-Met-enkephalinamide. J Neurochem 38:1336-1341

Grahame-Smith D.G. (1971) Studies *in vivo* on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. J Neurochem 18:1053-1066

Gudelsky G.A. and Porter J. (1979) Morphine- and opioid peptide-induced inhibition of the release of dopamine from tuberoinfundibular neurons. Life Sci 25:1697-1702

Heller H. C. (1979) Hibernation: neural aspects. Ann Rev Physiol 41:305-321

Herkenham M. and Mclean S. (1988) The anatomical relationship of opioid peptides and opioid receptors in the hippocampi of four rodent species. Opioids in the hippocampus McGinty J.F. and Friedman D.P. (eds), pp 33-47, NIDA publicantion, Rockville.

Ho B. Y. and Takemori A. E. (1989) Serotonergic involvement in the antinociceptive action of and the development of tolerance to the *kappa*-opioid receptor agonist, U50,488H. J Pharmacol Exp Ther 250 508-514

Illes P. (1989) Modulation of transmitter and hormone release by multiple neuronal opioid receptors. Rev Physiol Biochem Pharmacol 112:139-233

Itoh J., Ukai M., and Kameyama T. (1994) Dynorphin A-(1-13)) potently improves the impairment of spontaneous alteration performance induced by the μ-selective opioid receptor agonist DAMGO in mice. J Pharmacol Exp Ther 269:15-21

Izquierdo I. (1990) Acetylcholine release is modulated by different opioid receptor types in different brain regions and species. TIPS 11:179-180

Jackisch R., Geppert M., Lupp A., Huang H.Y., and Illes P. (1988) Types of opioid receptors modulating neurotransmitter release in discrete regions. Regulatory roles of opioid peptides. Illes P. and Farsang C. (eds),pp 240-258, VCH, Weinheim.

Jhamandas K., Horn V., and Sutak M. (1975) Comparative effects of opiate agonists methadone, levorphanol and their isomers on the release of rat cortical Ach *in vivo* and *in vitro*. Can J Physiol Pharmacol 53:540

Jhamandas K., Sawynok J., and Sutak M. (1977) Enkephalin effects on release of brain acetylcholine. Nature 269:433-434

Jin Z.L., Lee T.F., Zhou S.J., and Wang L.C.H. (1993) Age-dependent changes in the inhibitory effect of an adenosine agonist on hippocampal acetylcholine release in rats. Brain Res Bull 30:149-152

Kalén P., Strecker R.E., Rosengren E., and Björklund A. (1988) Endogenous release of neuronal serotonin and 5-hydroxyindoleacetic acid in the caudate-putamen of the rat as revealed by intracerebral dialysis coupled to high-performance liquid chromatography with fluorometric detection. J Neurochem 51:1422-1435

Kramarova L.I., Kolaeva S.G., Yukhanaov R.Y., and Rozhanets V.V. (1983) Content of DSIP, enkephalins, and ACTH in some tissue of active and hibernating ground squirrels. Comp Biochem Physiol 74C:31-33

Lee H.K., Dunwiddie T., and Hoffer B. (1980) Electrophysiological interactions of enkephalins with neuronal circuity in the rat hippocampus II. effects on interneuron excitability. Brain Res 184:331-342

Lipton P. (1985) Brain Slices: uses and abuses. Neuromethods 1. General neurochemical techniques. Boulton A.A. and Baker G.B. (eds), pp 69-116, Humana Press, Clifton.

Loh H.H., Brase D.A., Sampath-Khanna S., Mar J.B., Way E.L., and Li C.H. (1976) β-endorphin: *in vitro* inhibition of striatal dopamine release. Nature 264:567-568

Lubetzki C., Chesselet M.F., and Glowinski J. (1982) Modulation of dopamine release in the rat striatal slices by delta opiate agonists. J Pharmacol Exp Thr 222:435-440

Mansour A., Khachaturian H., Lewis M.E., Akil H., and Watson S.J. (1988) Anatomy of CNS opioid receptors. TINS 11:308-314

Mansour A., Fox C.A., Burke S., Meng F., Thompson R.C., Akil H., and Eatson S.J. (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an *in situ* hybridization study. J Comp Neurol 350:412-438

Matos F.F., Rollema H., and Basbaum A.I. (1990) Characterization of monoamine release in the lateral hypothalamus of awake, freely moving rats using *in vivo* microdialysis. Brain Res 528:39-47

Matsumoto M., Yoshioka M., Togashi H., Hirokami M., Tochihara M., Ikeda T., Smith C., and Saito H. (1994) *Mu*-opioid receptors modulate noradrenaline release from the rat hippocampus as measured by brain microdialysis. Brain Res 636:1-8

Millan M. J. and Colpaert P. C. (1990) Attenuation of opioid induced antinociception by 5-HT$_{1A}$ partial agonists in the rat. Neuropharmacology 29:315-318

Moore R.Y., Halaris A.E., and Jones B.E. (1978) Serotonin neurons of the midbrain raphe: ascending projections. J Comp Neurol 180:417-438

Mulder A.H., Wardeh G., Hogenboom F., and Frankhuyzen A.L. (1989) Selectivity of various opioid peptides towards *delta-*, *kappa*, and *mu*-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the brain. Neuropeptides 14:99-104

Mulder A.H., Schoffelmeer A.N.M., and Stoof J.C. (1990) On the role of adenylate cyclase in presynaptic modulation of neurotransmitter release mediated by monoamine and opioid receptors in the brain. Ann N Y Acad Sci 604:237-249

Nicoll R.A., Siggius G.R., Ling N., Bloom F.E., and Guillemin R. (1977) Neuronal actions of endorphins and enkephalins among brain regions: a comparative microinophoretic study. Pro Natl Acad Sci USA 74:2584-2588

North R.A., Williams J.T., Surprenant A., and Christie M.J. (1987) *mu* and *delta* receptors belong to a family of receptors that are coupled to potassium channels. Proc Natl Acad Sci USA 84:5487-5491

Novotona R., Jansky L., and Drahota Z. (1975) Effect of hibernation on turnover of serotonin in the brain stem of golden hamster (*Mesicricetus auratus*). Gen Pharmacol 6:23-26

Nurnberger F, Lee T. F., Jourdan M. L., and Wang L. C. H. (1991) Seasonal Changes in methionine-enkephalin immunoreactivity in the brain of hibernator, *Spermophilus columbianus*. Brain Res 547:115-121

Nurnberger F., Lee T.F., Staiger J.F., and Wang L.C.H. (1994) Involvement of limbic-neuroendocrine interactions in control of hibernation. Integrative and cellular aspects of autonomic functions: temperature and osmoregulation Pleschka K. and Gerstberger R. (eds), pp 259-268, John Libbey Eurotext, Paris.

Oka T. (1978) 5-HT and narcotic-induced hypothermia. Gen Pharmacal 9:151-154

Pakhotin P.I., Pakhotina D.I. and Belousov B. (1993) The study of brain slices from hibernating mammals *in vitro* and some approaches to the analysis of hibernation problems *in vivo*. Prog Neurobiol 40:123-161

Passarelli T. and Costa T. (1988) *Mu* and *delta* opioid receptors inhibit serotonin release in Rat Hippocampus. J Pharmacol Exp Ther 248:299-305

Popova N.K., Voronova I.P., and Kulikov A.V. (1993) Involvement of brian tryptophan hydroxylase in the mechanism of hibernation. Pharmacol Biochem Behav 46:9-13

Robert J.J., Orosco M., Rouch C., Jacquot C., and Cohen Y. (1989) Effects of opiate agonists and an antagonist on food intake and brain neurotransmitters in normophagic and obese "cafeteria" rats. Pharmacol Biochem & Behav 34:577-583

Robert J.J., Orosco M., Rouch C., Cohen Y., and Jacquot C. (1991) Effects of dexfenfluramine and opioid peptides, alone or in combination, on food intake and brain serotonin turnover in rats. Pharmacol Biochem & Behav 38:775-580

Sawyrok J. (1989) The 1988 Merck Frosst Award. The role of ascending and descending noradrenergic and serotonergic pathways in opioid and non-opioid antinociception as revealed by lesion studies. Can J Physiol Pharmacol 67:975-988

Segal M. (1975) Physiological and Pharmacological evidence for a serotonergic projection to the hippocampus. Brain Res 94:115-131

Sharif N.A. and Hughes J. (1989) Discrete mapping of brain *mu* and *delta* opioid receptors using selective peptides: quantitative autoradiography, species differences and comparison with *kappa* receptors. Peptides 10:499-522

Sharp T., Bramwell S.R., Clark D., and Grahame-Smith D.G. (1989) *In vivo* measurement of extracellular 5-hydroxytryptamine in hippocampus of the anaesthetized rat using microdialysis: changes in relation to 5-hydroxytryptaminergic neuronal activity. J Neurochem 53:234-240

Simmons M.L., Wagner J.J., Caudle R.M., and Chavkin C. (1992) Endogenous opioid regulation of norepinephrine release in guinea pig hippocampus. Neurosci Lett 141:84-88

Simon E.J. (1991) Opioid receptors and endogenous opioid peptides. Med Res Rev 11:357-374

Simonds W.F. (1988) The molecular basis of opioid receptor function. Endocrine Rev 9:200-212

Siniscalchi A., Bertelli M.G., Bianchi C., and Beani L. (1990) Effect of opioid antagonists on acetylcholine release from guinea-pig brain slices: influence of peptidase inhibition. Pharmac Res 22:493-501

Spafford D.C. and Pengelley E. T. (1971) The influence of the neurohumoral serotonin on hibernation in the golden-mantled ground squirrel, *Citellus lateralis*. Comp Biochem Physiol 38A:239-250

Stengaard-Pedersen K. (1989) Opioid Peptides and Receptors Progress in Histochemistry and Cytochemistry. Vol.20, No.3, G Fischer, Stuttgart.

Taube H.D., Borowski E., Endo T., and Starke K. (1976) Enkephalin: a potential modulator of noradrenaline release in rat brain. Eur J Pharmacol 38:377-380

Traynor J.R. and Elliott J. (1993) *Delta*-opioid receptor subtypes and cross-talk with *mu*-receptors. TIPS 14:84-86

Ueda H., Misawa H., Katada T., Ui M., Takagi H., and Satoh M. (1989) Distinct modes of coupling of *mu-*, *delta-*, and *kappa*-opioid receptors with GTP-binding proteins in brain membranes. Progress in Opioid Research Advances in the Biosciences Vol.75, Cros J., Meunier J.C., and Hamon M. (eds), pp 129-132, Pergamon Press, New York.

Ueda H., Nozaki M., and Satonh M. (1991) Multiple Opioid Receptors and GTP-Binding Proteins. Comp Biochem Physiol 98C:157-169

Ungerstedt U. (1991) Introduction to intracerebral microdialysis. Microdialysis in the Neurosciences. Robinson T.E. and Justice J.B.(eds), pp 3-22, Elsevier, Amsterdam.

Van Loon G.R. and Souza E.B. (1978) Effects of β-endorphin on brain serotonin metabolism. Life Sci 23:971-978

Wagner J.J., Caudle R.M., and Chavkin C. (1992) Kappa-opioids decrease excitatory transmission in the dentate gyrus of the guinea pig hippocampus. J Neurosci 12:132-141

Westerink B.H.C. and Justice J.B. (1991) Microdialysis compared with other in vivo release models. Microdialysis in the Neurosciences. Robinson T.E. and Justice J.B. (eds), pp 23-46, Elsevier, Amsterdam.

Wollemann M. (1990) Recently developments in the research of opioid receptor subtype molecular characterization. J Neurochem 54:1095-1101

Wollemann M., Benyhe S., and Simon J. (1993) The kappa-opioid receptor: evidence for the different subtypes. Life Sci 52:599-611

Wood P.L. (1986) Multiple opioid receptors in the central nervous system. Neuromethods:4. Receptor Binding Boulton A.A., Baker G.B., Hrdina P.D. (eds), pp 329-363, Humana Press, Clifton.

Yoshioka M., Matsumoto M., Togashi H., Smith C.B., and Saito H. (1993) Opioid receptor regulation of 5-hydroxytryptamine release from the rat hippocampus measured by in vivo microdialysis. Brain Res 613:74-79

Zielgansberger W., French E.D., Siggius G.R., and Bloom F.E. (1979) Opioid peptides may excite hippocampal pyramidal neurons by inhibiting adjacent inhibitory interneurons. Science 205:415-417

CHAPTER THREE

Changes in the thermoregulatory effects of opioids and 5-HT in the septo-hippocampal complex in the hibernation cycle

Introduction

Among the many physiological functions of opioids, their thermoregulatory effects have been substantially investigated (for reviews see Clark 1983, Clark and Lipton 1983, 1991, Myers and Lee 1989, Adler and Geller 1992). The alteration of body temperature (Tb), following administration of opioid ligands, varies depending on the specificity of ligands, dosage, route of application, species, ambient temperature, and the physiological condition of the animal used (Wang et al. 1987, Adler et al. 1988, Shukla and Dhawan 1988, Burks 1991, Handler et al. 1992, 1994). For instance, intracerebroventricular (icv) injection of a μ selective ligand, DAGO, caused hyperthermia in rats, while injection of a δ ligand, DPDPE and a κ ligand, U50488, induced hypothermia (Spencer et al. 1990). In rats, subcutaneous injection of morphine, a ligand with some μ potency, caused an increase in Tb at low dose and a decrease in Tb at high dose, whereas icv administration of morphine induced only hyperthermia at both low and high doses (Adler et al. 1988). When rats were restrained, icv application of the μ agonist, DAGO, caused hypothermia rather than the hyperthermia observed in unrestrained rats (Spencer et al. 1988). Alteration of ambient temperature (Ta) from 30°C to 5°C also changed the hyperthermic response in rats to icv injection of DAGO to a hypothermic response (Handler et al. 1994). In ground squirrels, icv application of morphine produced a biphasic hyperthermia/hypothermia thermoregulatory response in the non-hibernating phase; however, in the hibernating phase, morphine provoked only hyperthermia even at high dose (Wang et al. 1987).

Other than the icv approach, the role of opioids on the central nervous system (CNS) thermoregulation has been mainly limited to the preoptic area and anterior hypothalamus (POAH, Adler et al. 1988, Burks 1991, Clark and Lipton 1991). The possible role of opioids in modulating thermoregulation in other CNS regions has not been systematically studied. The hippocampus and septum have been implicated in the integration of thermal stimuli and are involved in thermoregulatory functions (Murakami et al. 1984, Boulant et al. 1989). The metabolic activity of the hippocampus increased during autonomic thermoregulation (Murakami et al. 1984). Electrical stimulation of the hippocampus in rats induced a change in Tb in a direction towards the ambient temperature (Osaka et al. 1984). Thermosensitive neurons have been shown to exist in the septum (Hori et al. 1989, Nakashima et al. 1989, Zeisberger 1990) and electrophysiological and neuroanatomical studies have demonstrated neuronal connections between the hypothalamus and the septo-hippocampal complex (Kiyohara et al. 1984, Disturnal et al. 1986). Intraseptal administration of opioid agonists, morphine and D-Ala^2-met-enkephalinamide, evoked a biphasic change in Tb in non-hibernating ground squirrels (Lee et al. 1989). Therefore, the septo-hippocampal complex may also be a potential neuronal structure involved in thermoregulation.

The monoamine 5-HT has long been known as one of the important CNS neurotransmitters involved in thermoregulation (Komiskey and Rudy 1977, Bligh 1979, Myers and Lee 1989, Chopin et al. 1994). It has been demonstrated that depletion of central 5-HT by p-chlorophenylalanine (p-CPA) attenuates the hypothermic effect caused by i.p. morphine administration (Haubrical and Blake 1971). In addition, application of an exogenous 5-HT precursor to p-CPA-treated animals could restore morphine-elicited hypothermia (Oka 1977). Lesioning of the raphe nuclei, a major 5-HT source, also abolished morphine-mediated hypothermia (Samanin and Garattini 1972). Thus, there is evidence that 5-HT may mediate the morphine-induced thermoregulatory response (Oka 1978).

Since hibernation is considered a modified form of thermoregulation (Florant and Heller 1977, Florant et al. 1978, Jansky 1990) and both opioids and 5-HT have been shown to influence thermoregulation, it is possible that opioids and 5-HT may be involved in the regulation of hibernation by interacting with each other. The present study investigated the thermoregulatory functions of opioids and 5-HT in the septo-hippocampal regions in different phases of a hibernation cycle. The interaction of opioids and 5-HT on the thermoregulatory response was also examined.

Methods

All experimental procedures received prior approval of the University of Alberta Animal Use Committee and followed the guidelines of the Canadian Council on Animal Use.

Animals Columbian ground squirrels (*Spermophilus columbianus*) of both sexes were live trapped in the Rocky Mountains of Alberta. Squirrels were housed individually at 22°C under natural photoperiod and supplied with food (rodent chow, mixed grains, and sunflower seeds) and water *ad lib.* Body weights of the squirrels were measured year round on a weekly basis. Onset of the hibernation season in an animal was indicated by a rapid weight gain, followed by a weight plateau and anorexia. When the body weight reached the plateau, the animal was transferred to a cold (4±1°C), dark, walk-in environmental chamber to facilitate hibernation. The activity of the animal was monitored daily. Hibernation was characterized by a the lack of movement and extremely slow, and regular breathing (less than one per minute). The duration of hibernation was estimated using the sawdust method: sawdust placed on the back of the animal remained on the following day if the animal was hibernating. Non-hibernating animals were characterized by having relatively stable body weights for at least two months before their use.

Cannulation of the septal and hippocampal regions Ground squirrels in the hibernating phase were cannulated only when they had completed two hibernation bouts and were in an interbout euthermic period. Under halothane anaesthesia, the skull basal plane of ground squirrels was first adjusted horizontally by tilting the upper incisor down D x Sin35° mm, where D is the distance from the interaural bar to the incisor. Using the interaural bar as the zero stereotaxis point, the coordinates for the septum were AP =13 X CR mm, L =0.7 X CR mm, H =4 X CR mm and those for the hippocampus were AP =10 X CR -1 mm, L =4 X CR mm, H =3 X CR mm. The CR, correction factor, was D divided by 36.8 mm. The tip of a 23-gauge stainless steel guide cannula was bevelled and placed 1 mm above the intended injection site and secured with dental cement on the skull of the animal. A 27-gauge stainless steel stylet was always kept in the guide cannula when the cannula was not in use. The exact anatomical location of the injection site was verified histologically following completion of the experiments. At the time of cannula implantation, a pre-calibrated temperature-sensitive radiotransmitter (Model T-M, Mini-Mitter Co.) was placed in the peritoneal cavity. Non-hibernating ground squirrels were used one week after surgery. Hibernating animals were not used until they had again hibernated for two days and then were artificially aroused before experimentation (body temperature, Tb=37°C) by transferring them to room temperature (22°C) for about six hours.

Determination of body temperature and metabolic rate The squirrel was placed in a water-jacketed Plexiglas metabolic chamber (20 x 20 cm, diameter x height) in which the ambient temperature could be controlled accurately at 20 ± 1°C or

5 ± 1°C. Once the animal was in the chamber, it was not handled again throughout the experiment. Using a mass-flow controller (Matheson model 8240 controller and 8142 transducer), dry air to the metabolic chamber was regulated at 1500 ml/min STP. The oxygen and carbon dioxide concentration of the exhaust gas, from the metabolic chamber, was analyzed simultaneously by splitting it into two streams. Oxygen concentration was measured by an O_2 analyzer (Applied Electrochemistry, S-3A/II) after desiccation with Drierite and removal of CO_2 by Ascarite. The concentration of carbon dioxide was measured using a CO_2 analyzer (Applied Electrochemistry, CD-3A) after desiccation. The animal's Tb was determined and recorded every minute with the radiotransmitter implanted in the animal. The signals from the O_2 analyzer, CO_2 analyzer, and telemeter receiver were processed by an IBM personal computer interfaced with an ISAAC 91-I data acquisition system. Oxygen consumption ($\dot{V}O_2$) and respiratory quotient (RQ) were calculated using the method of Wang (1978). The heat production (HP) was calculated using Kleiber's equation (1975):

$$HP = \frac{87 + 27RQ}{22.4} \times \dot{V}O_2$$

Thermoregulatory effect of drugs after central administration Prior to administration of drugs, the animal was kept in the metabolic chamber for at least 60 minutes or until its Tb and metabolic HP had stabilized. Each drug used was freshly prepared in artificial CSF (NaCl 128 mM, KCl 2.55 mM, $CaCl_2$ 1.26 mM, $MgCl_2$ 0.94 mM) and passed through a 0.22 mm Swinnex Millipore filter to eliminate pyrogens and bacteria before injection. Opioid agonists or 5-HT (in 1 μl artificial CSF) at various concentrations were injected into the cannulated areas via a 27-gauge needle. Cyproheptadine was injected intraperitoneally 15 minutes before the central administration of opioids or 5-HT at a dose of 2.5 mg(ml·kg)$^{-1}$ weight. The behaviour of the animal was closely observed throughout the entire experimental period. To avoid inducing physical dependence to opioids in the ground squirrels, the animals were used only every other day.

Statistics Body temperature and HP of Columbian ground squirrels in the-non-hibernating and hibernating phases before injection were compared using Student's t-test. The effects of drugs on Tb and HP over time were analyzed using one-way ANOVA by comparing post-injection values with pre-injection levels and two-way ANOVA by comparing the post-injection changes following drug administration with vehicle injection. Significance was set at $P < 0.05$.

Chemicals [D-Ala^2]-Met-Enkephalinamide, 5-hydroxytryptamine (5-HT) creatinine sulphate, and cyproheptadine were obtained from Sigma. U50488 was purchased from Research Biochemicals Inc. Others were products of BDH.

Results

Thermoregulatory effect of 5-HT in the hibernation cycle

The average Tb of ground squirrels in the non-hibernating phase at a Ta of 20 °C, before drug applications, was 37.55 ± 0.32°C (n=8). Their HP was 2.09 ± 0.10 Kcal/(Kg·h)$^{-1}$ (8.74 ± 0.42 KJ(Kg·h)$^{-1}$). Intra-septal injection of artificial CSF did not have significant impact (P=0.90) on the Tb of the animals (Figure III.1a). Heat production rose slightly, but, not significantly (P=0.54) within the first 20 minutes after CSF injection . Intra-septal application of 5-HT creatinine sulphate at 12.91 n mol to 51.63 n mol induced a suppression in HP, initiated at 10 minutes post-injection and reached maximum at 15 to 30 minutes (Figure III.1b) (two-way ANOVA in comparison to CSF administration, $F_{1,247}=8.69$, $P<0.01$ for 12.91 n mol 5-HT and $F_{1,228}=6.92$, $P=0.01$ for 51.63 n mol 5-HT). A dose-dependent hypothermic effect was observed following the decline in HP.

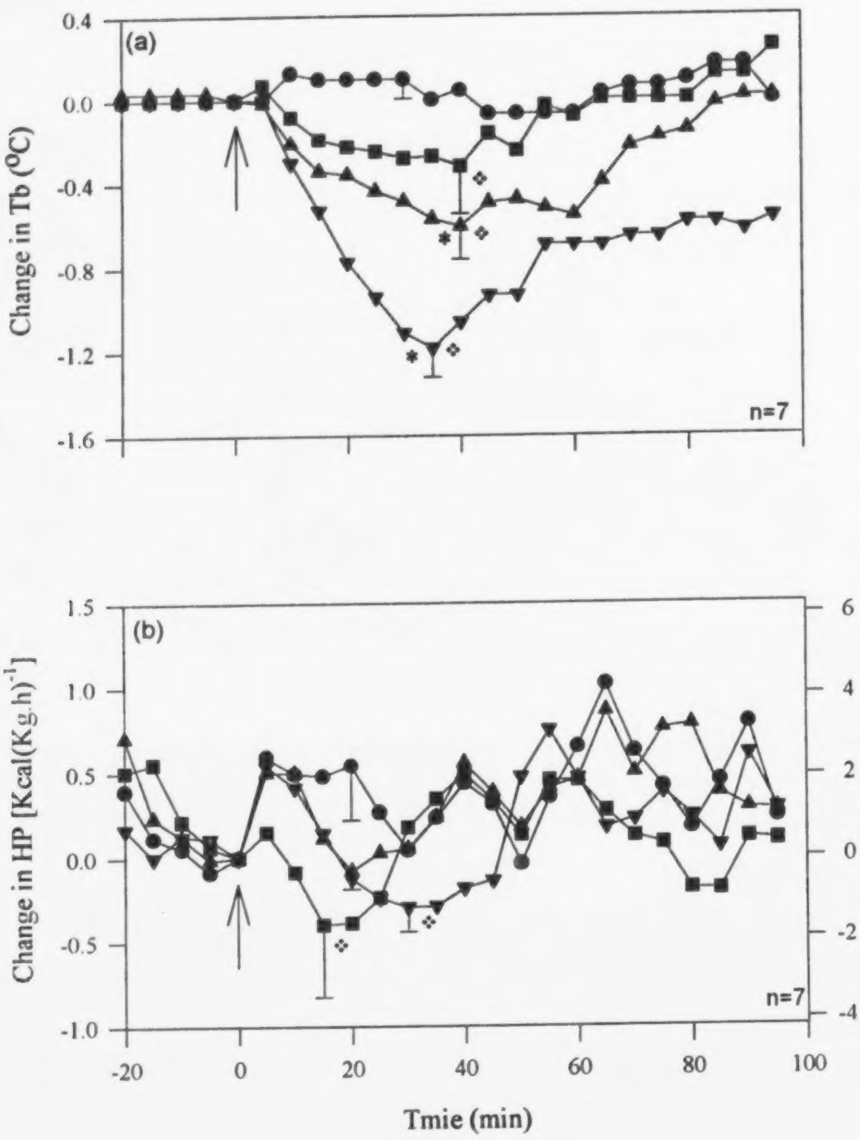


Figure III.1 Thermoregulatory effect after lateral septal injection of 5-HT in non-hibernating ground squirrles at 20°C. (Arrow indicates injection of CSF: closed circle; 12.91 n mol 5-HT: closed square; 25.81 n mol 5-HT: closed triangle; 51.63 n mol 5-HT: closed reversed triangle). * means change in Tb following injection is significantly different from its pre-injection values, whereas ❖ indicates the change is significantly different from the vehicle (CSF) treatment.

The decrease in Tb maximized between minute 30 and 40 after 5-HT injection (Figure III.1a). There was also a moderate increase in exploring activity of the ground squirrel within the first 10 minutes following injection. The animal then spread out and rested for the next 20 minutes or so; after which its activity returned to normal. Although the drop in Tb, comparing with pre-injection level, was significant only following injections of 25.81 and 51.63 n mol 5-HT (0.61°C, $P<0.01$ and 1.18°C, $P<0.01$, respectively), the Tb changes following application of 5-HT at all three doses was significantly different from that after CSF injection (two-way ANOVA, main effect of treatment compared to CSF, $F_{1,247}=6.04$, $P=0.02$ for 12.91 n mol 5-HT; $F_{1,247}=67.59$, $P<0.01$ for 25.81 n mol 5-HT; and $F_{1,228}=261.76$, $P<0.01$ for 51.63 n mol 5-HT). The decrease in Tb seemed to be at least partly the result of the suppression in HP because the reduction in HP always occurred and reached maximum prior to that of Tb.

When non-hibernating ground squirrels were held at a Ta of 5°C, average Tb (37.48 ± 0.35°C, n=8) was maintained at the same level as when they were at 20°C ($P=0.89$) through increased heat production (3.10 ± 0.19 $\mathrm{Kcal(Kg\cdot h)^{-1}}$ or 12.96 ± 0.79 $\mathrm{KJ(Kg\cdot h)^{-1}}$, $P<0.01$). The decline in Tb following 5-HT administration at Ta=5°C (0.54, 0.70, and 1.49 for the same three doses; see P.66) was slightly greater and maximized sooner than that seen at 20°C (Figure III.2a). At all three doses used, a profound decline in HP comparing with pre-injection ($P<0.01$ for 12.91 n mol 5-HT, $P=0.02$ for 25.81 n mol 5-HT, and $P=0.03$ for 51.63 n mol 5-HT) accompanied and preceded the significant decrease in Tb (Figure III.2).

Following disturbed arousal, ground squirrels in their hibernating phase were kept in the metabolic chamber at 5°C, maintained their Tb at the same level as those in the non-hibernating phase (37.47 ± 0.30°C, $P=0.98$) (n=8). Similarly, HP was also close to those in the non-hibernating phase (3.00 ± 0.18 $\mathrm{Kcal(Kg\cdot h)^{-1}}$ or 12.54 ± 0.75 $\mathrm{KJ(Kg\cdot h)^{-1}}$, $P=0.71$). Following CSF administration,

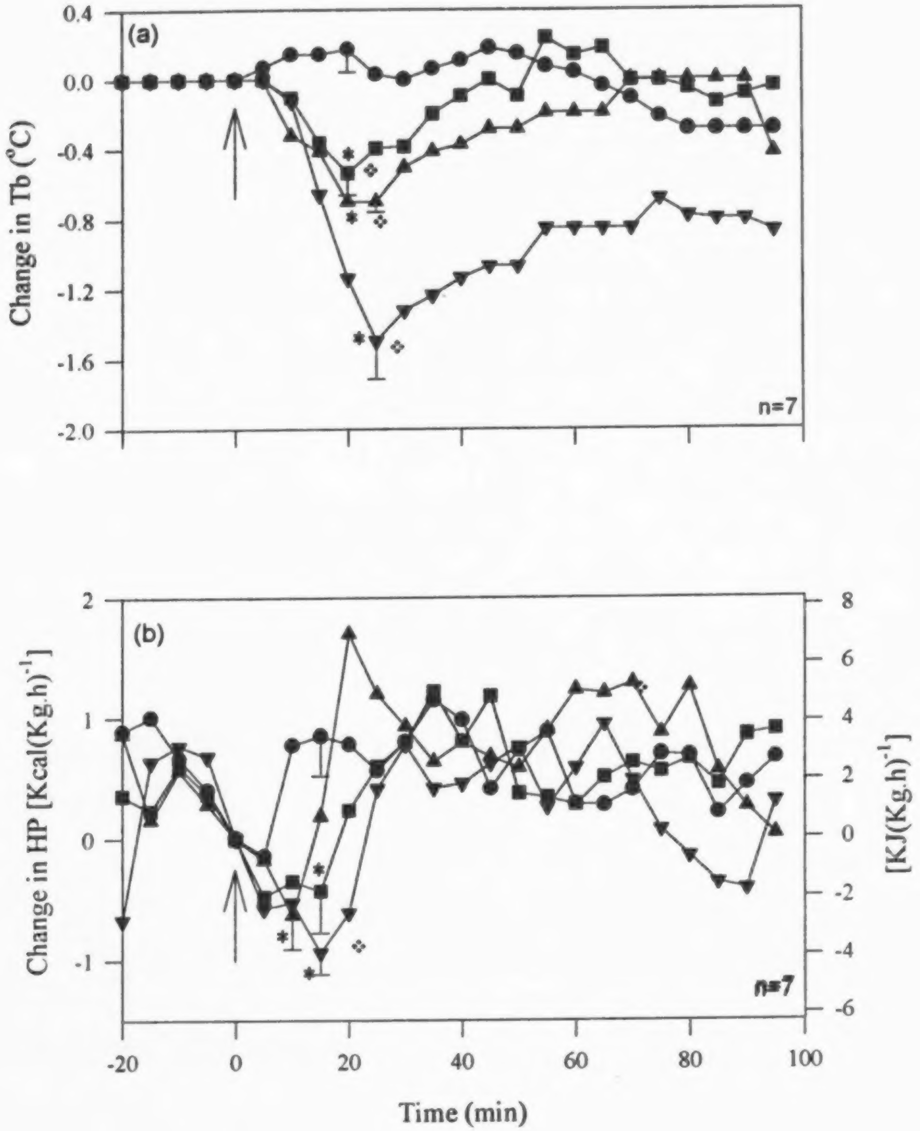


Figure III.2 Thermoregulatory response following lateral septal administration of 5-HT in non-hibernating ground squirrles at 5°C. (Arrow indicates administration of CSF; closed circle; 12.91 n mol 5-HT; closed square; 25.81 n mol 5-HT; closed triangle; 51.63 n mol 5-HT; closed reversed triangle). * represents significantly different from its pre-treatment level, while ❖ indicates significantly different from the control (CSF) treatment.

the change in Tb did not differ from that in the non-hibernating phase (two-way ANOVA main effect of state, $F_{1,253}=0.63$, $P=0.43$). However, intra-septal injection of the same doses of 5-HT did not produce as profound a thermoregulatory effect as that seen in the non-hibernating phase. As indicated in Figure III.3, only at 51.63 n mol 5-HT did a significant reduction in Tb (0.56°C, $P<0.01$, comparing with pre-injection level and CSF injection) occur and which was significantly smaller than that seen in the non-hibernating phase (two-way ANOVA main effect of state, $F_{1,276}=11.71$, $P<0.01$). The suppression of HP after 5-HT injection was also only significant at 51.63 n mol ($P=0.04$).

Figure III.4 indicates the anatomic maps of the injection sites. Closed circles represent the sites at which 5-HT administration induced significant hypothermia. Only these sites, which were mainly located in the lateral septum, were used to evaluate the thermoregulatory responses. Injection of 5-HT to the medial septum or caudate nucleus putamen (open circles) did not evoke a hypothermic response and was not included in calculating thermoregulatory responses.

Possible Interaction of 5-HT and opioids in thermoregulation in the septal region

Previous experiments have demonstrated that intra-septal administration of high dose of [D-Ala^2]-Met-Enkephalinamide also induced hypothermia in non-hibernating ground squirrels (Lee et al. 1989). Therefore, the possibility that opioids provoked hypothermia by interacting the serotonergic system was investigated. As shown in Figure III.5, both 51.63 n mol of 5-HT and 87.92 n mol of met-enkephalinamide, injected into the lateral septum, resulted in a profound hypothermia in the non-hibernating ground squirrels ($P<0.01$ and $P=0.03$, respectively). The drop in Tb, induced either by 5-HT or enkephalinamide, could be attenuated by pretreatment of a 5-HT antagonist cyproheptadine (2.5 mg/kg, i.p., $P=1.00$ and $P=0.86$, respectively). Cyprohaptidine alone did not induce any significant thermoregulatory effect

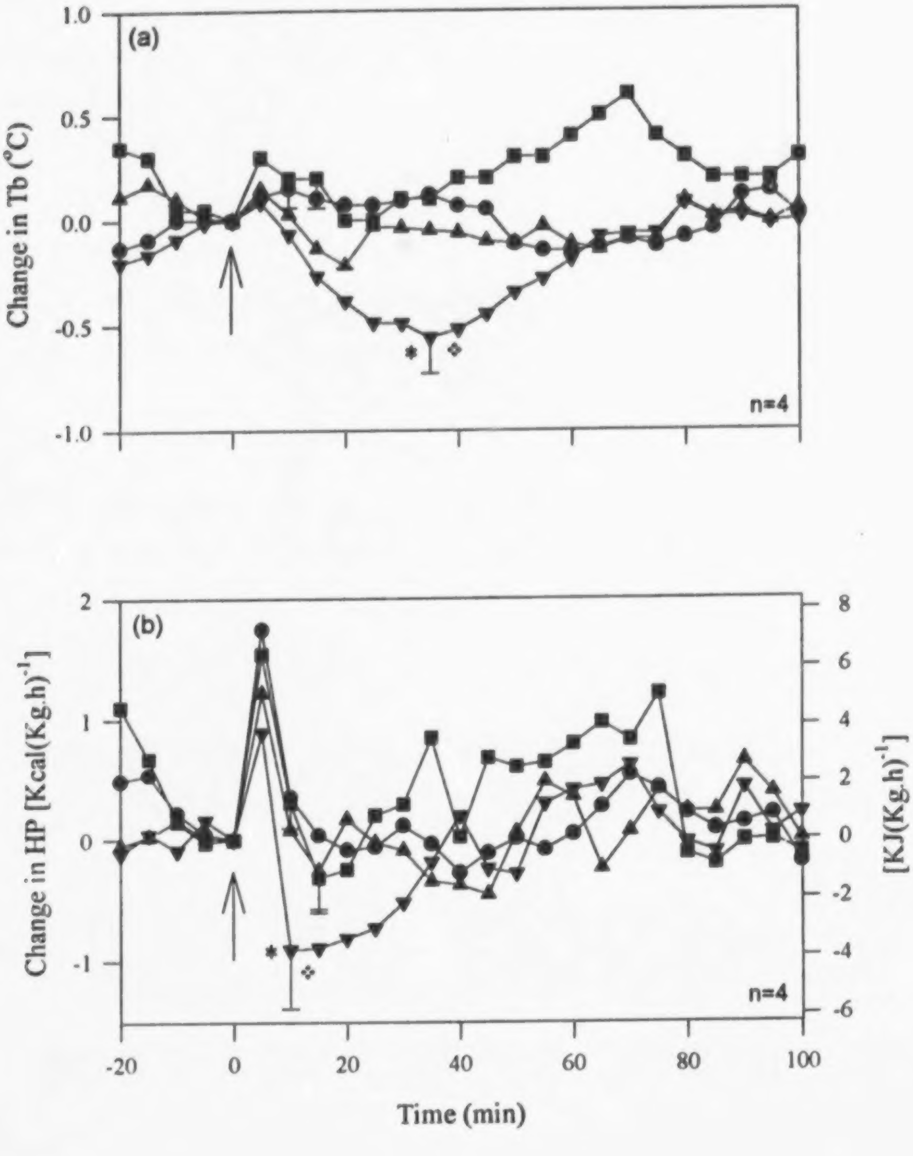


Figure III.3 Thermoregulatory effect after lateral septal injection of 5-HT in the hibernating phase at 5°C. (Arrow indicates application of CSF; closed circle; 12.91 n mol 5-HT; closed square; 25.81 n mol 5-HT; closed triangle; 51.63 n mol 5-HT; closed reversed triangle). * significantly different from its pre-treatment value; ❖ significantly different from the CSF control.

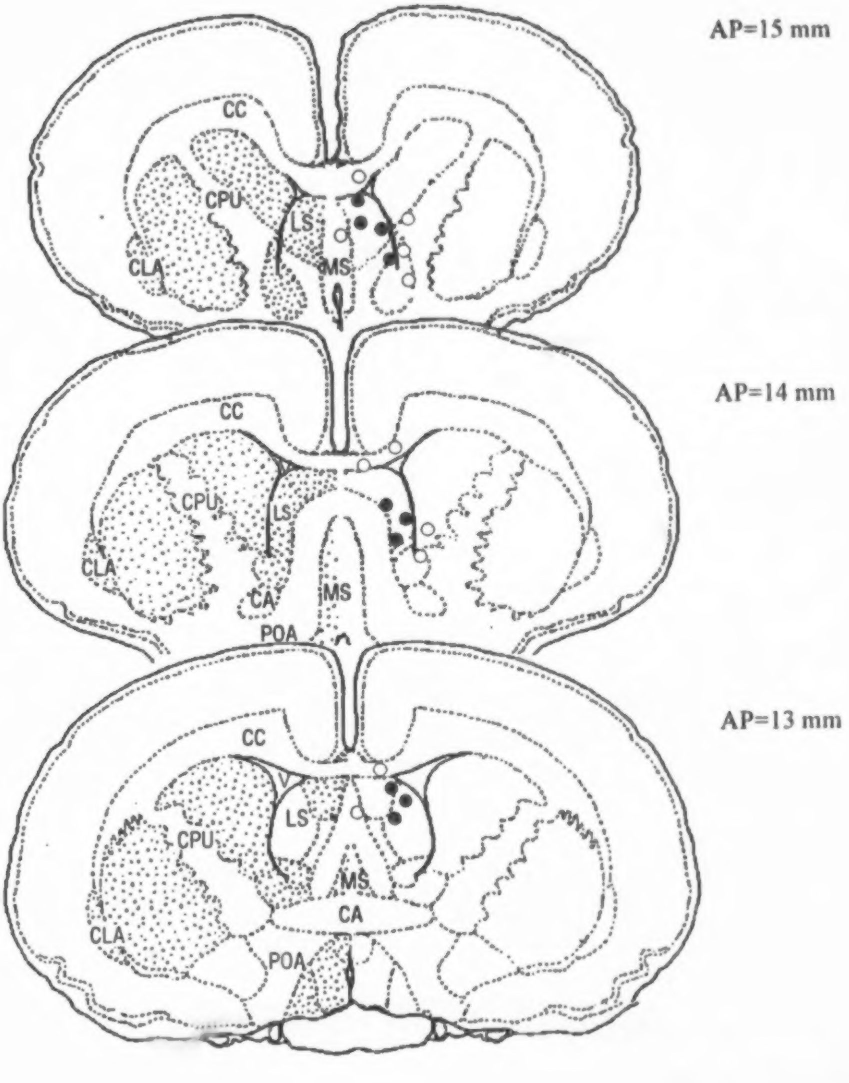

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Figure III.4 Stereotaxic loci of 5-HT administration in the septal region of Columbian ground squirrles. (closed circles represent the sites at which 5-HT elicited a dose-dependent hypothermia; open circles indicate the locations that produced no obvious change in Tb; CA: Anterior commissure; CC: Corpus callosum; CLA: Claustrum; CPU: Caudate nucleus putmen; LS: Lateral seputm; MS: Medial septum; POA: Lateral preoptic area).

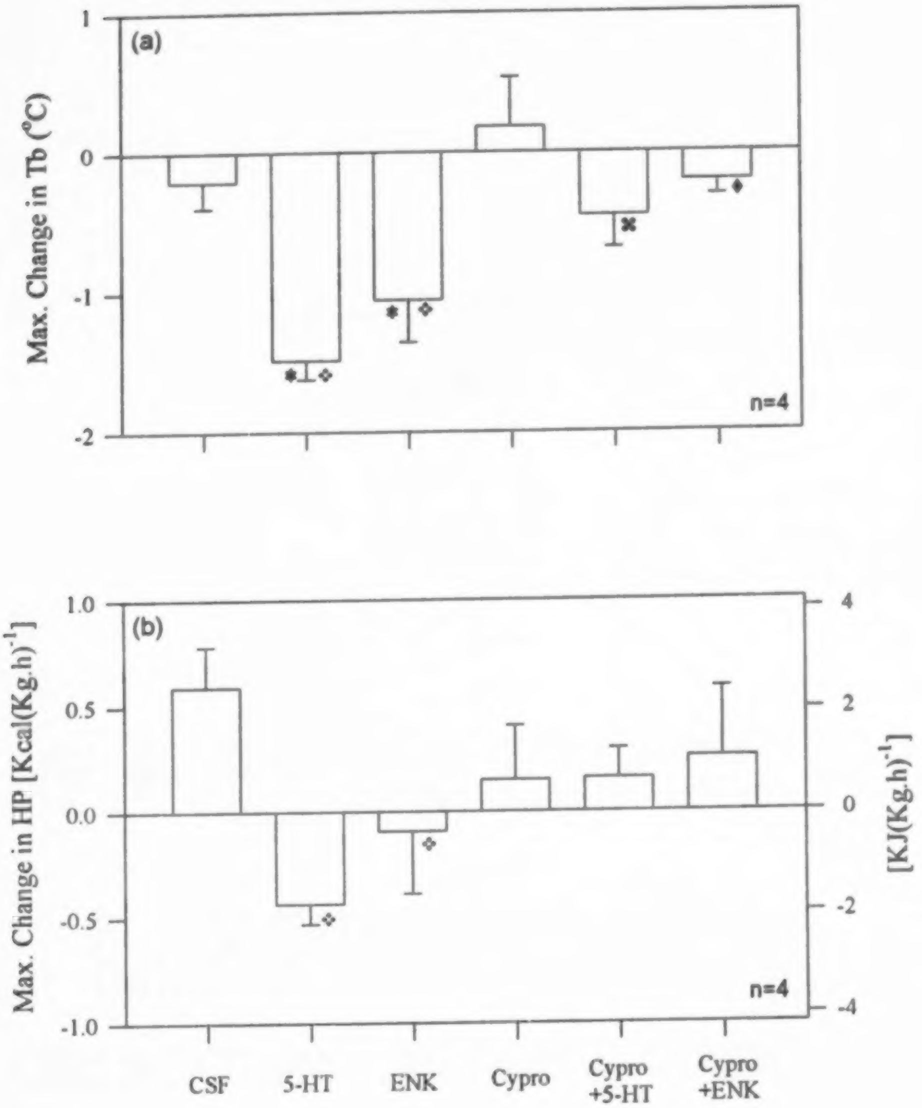


Figure III.5 Antagonism by cyprohaptidine (2.5 mg/Kg, i.p.) of hypothermia and HP suppression induced by lateral septal injection of 5-HT and met-enkephalinamide in non-hibernating ground squirrels. (5-HT: 51.63 n mol; met-enkephalinamide: 87.92 n mol). * significantly different from its pre-injection value; ❖ significantly different from the CSF control; ⌘ significantly different from 5-HT application; ◆ significantly different from ENK treatment.

(P=1.00). The change in Tb over time following 5-HT or enkephalinamide administration in cyprohaptidine-pre-treated animals was also different from 5-HT or enkephalinamide without the pre-treatment (two-way ANOVA, $F_{1,170}$=3.55, P=0.06 for 5-HT injection and $F_{1,161}$=6.20, P=0.01 for enkephalinamide injection).

Thermoregulatory effect of U50488 in the lateral septum

To examine the functional role of *k* receptors in thermoregulation, U50488 was injected into the lateral septal region of non-hibernating ground squirrels. In contrast to enkephalinamide, none of the three doses of U50488 (27.08, 54.17, and 108.33 n mol) elicited any significant thermoregulatory responses (Table III.1).

Effect of hippocampal administration of enkephalinamide and U50488 on body temperature of non-hibernating and hibernating ground squirrels

Injection of 1 ul of CSF into the hippocampus of non-hibernating ground squirrels, at 20°C, did not alter either Tb (P=1.00) or HP (P=0.98). Following intra-hippocampal injection of 3.52 n mol [D-Ala^2]-met-enkephalinamide, there was a slight increase in HP preceded an rise in Tb (Figure III.6). The elevation in Tb at 3.52 n mol was not significantly different from the pre-injection level (P=0.44), but the Tb change after opioid administration was significantly different from that of vehicle application (two-way ANOVA main effect of treatment, $F_{1,286}$=23.278, P<0.01). Administration of met-enkephalinamide at 17.58 n mol caused a significant increase in Tb (P<0.01) starting at minute 30 and lasting for more than 90 minutes (Figure III.6). The increase in Tb seemed to be related at least in part to an increase in HP (P=0.04). The changes in both Tb and HP at 17.58 n mol were significantly different from that of the CSF injection (two-way ANOVA, $F_{1,286}$=27.94, P<0.01 and $F_{1,286}$=18.14, P<0.01, respectively). An increase in grooming and exploring activity was also evident, especially in the first 30 minutes after opioid administration. In contrast, a

Table III.1 Thermoregulatory responses of non-hibernating Columbian ground squirrels to intra-septal and intra-hippocampal administration of U50488

	Max. Change in Tb (°C)	ANOVA P Value	Max. Change in HP (Kcal/kg/hr)	ANOVA P Value
Lateral Septum				
CSF	0.17 ± 0.16	0.96	0.88 ± 0.50	0.76
U50488, 27.08 nmol	-0.11 ± 0.09	0.79	0.74 ± 0.44	0.22
54.17 nmol	0.23 ± 0.33	1.00	0.67 ± 0.56	0.99
108.33 nmol	-0.17 ± 0.09	1.00	0.73 0.24	0.61
Hippocampus				
CSF	-0.18 ± 0.31	0.95	-0.48 ± 0.25	0.99
U50488, 27.08 nmol	-0.27 ± 0.27	0.85	0.67 ± 0.43	0.77
54.17 nmol	-0.19 ± 0.18	1.00	0.83 ± 0.46	1.00
108.33 nmol	-0.27 ± 0.18	1.00	0.57 ± 0.40	0.86

The results represent mean ± s.e. of four non-hibernating Columbian ground squirrels. ANOVA test was one-way.

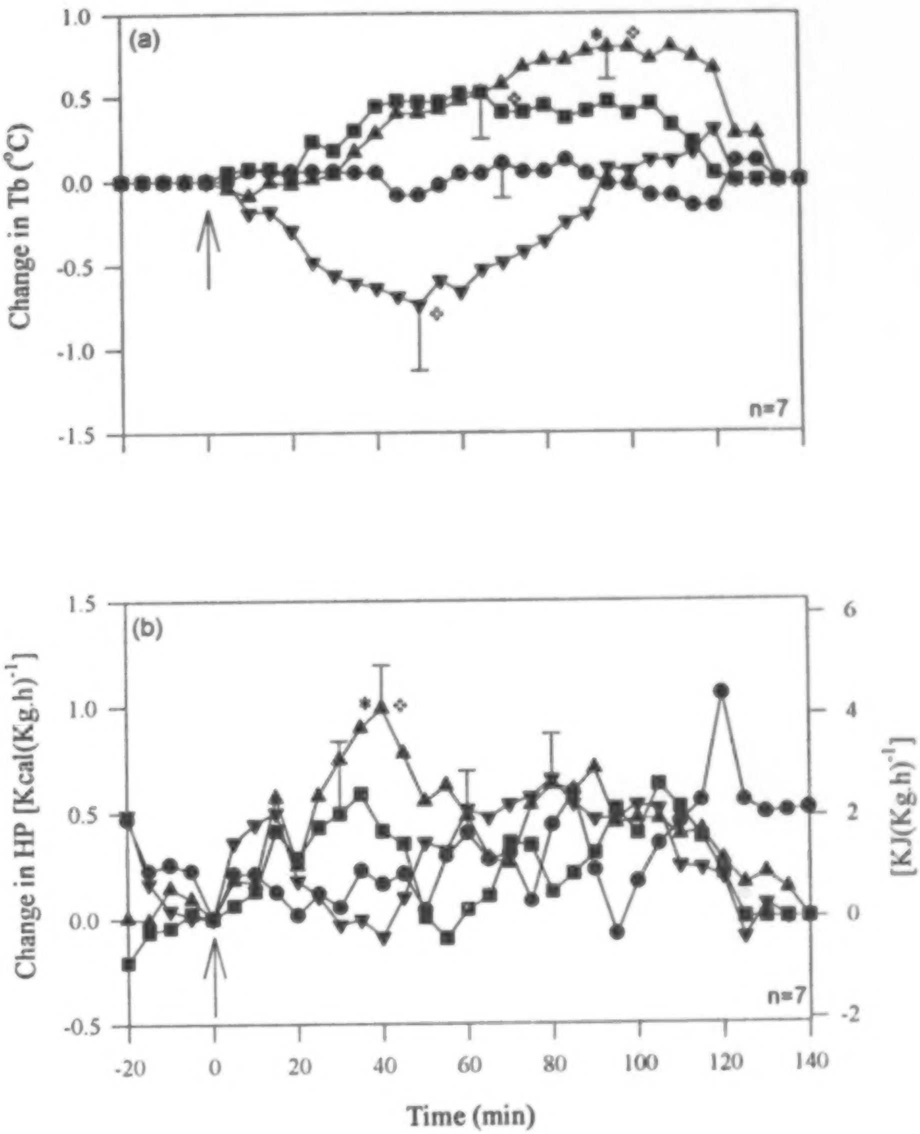


Figure III.6 Thermoregulatory effect of hippocampal administration of D-Ala^2-Met-enkephalinamide in non-hibernating ground squirrles at 20°C. (Arrow: injection; closed circle: CSF; closed square: 3.52 n mol ENK; closed triangle: 17.58 n mol ENK; closed reversed triangle: 87.92 n mol ENK). * significantly different from its pre-treatment value; ❖ significantly different from the vehicle (CSF) treatment.

higher dose (87.92 n mol) of met-enkephalinamide usually caused moving, licking, and finally spreading out on the floor of the chamber during the first 30 minutes. The high dose induced decline in Tb was not significant comparing with pre-injection level ($P=0.49$), but it significantly differed from the Tb change following CSF injection ($F_{1,284}=22.31$, $P<0.01$, two-way ANOVA). The decrease in Tb did not appear to be preceded by a reduction in HP (Figure III.6).

When the non-hibernating animals were exposed to 5 °C, intra-hippocampal administration of CSF did not influence their Tb or HP ($P=1.00$ and $P=0.98$ respectively, Figure III.7). Application of low doses (3.52 and 17.58 n mol) of met-enkephalinamide in the hippocampal region did not induce hyperthermia ($P=0.95$ and $P=0.99$ respectively and $F_{1,264}=2.40$, $P=0.12$, $F_{1,242}=2.22$, $P=0.14$ respectively, two-way ANOVA). At a high dose (87.92 n mol), however, met-enkephalinamide caused a significant decline in Tb comparing with the pre-injection level ($F_{28,202}=1.63$, $P=0.03$) and the vehicle injected value ($F_{1,286}=18.74$, $P<0.01$) (Figure III.7). Similar to the observation at 20°C, the hypothermia induced by this high dose of opioid could not be correlated with a suppression of HP.

When ground squirrels in the hibernating phase (artificially aroused) were held at 5°C, intra-hippocampal administration of met-enkephalinamide at low dose (3.52 n mol) did not have any effect on their Tb ($P=0.96$) (Figure III.8). In addition, high dose (87.92 n mol) of met-enkephalinamide induced a hyperthermic response (Figure III.8; two-way ANOVA main effect of treatment in comparison to CSF, $F_{1,198}=9.79$, $P<0.01$) rather than hypothermia observed in the non-hibernating animals. The change in thermoregulatory response to 87.92 n mol enkephalinamide was, therefore, state-dependent ($F_{1,335}=25.60$, $P<0.01$, two-way ANOVA, main effect of state). The rise in Tb following high dose of enkephalinamide in the hibernating phase possibly resulted from an increase in HP ($F_{1,198}=33.52$, $P<0.01$, two-way ANOVA), which was significantly

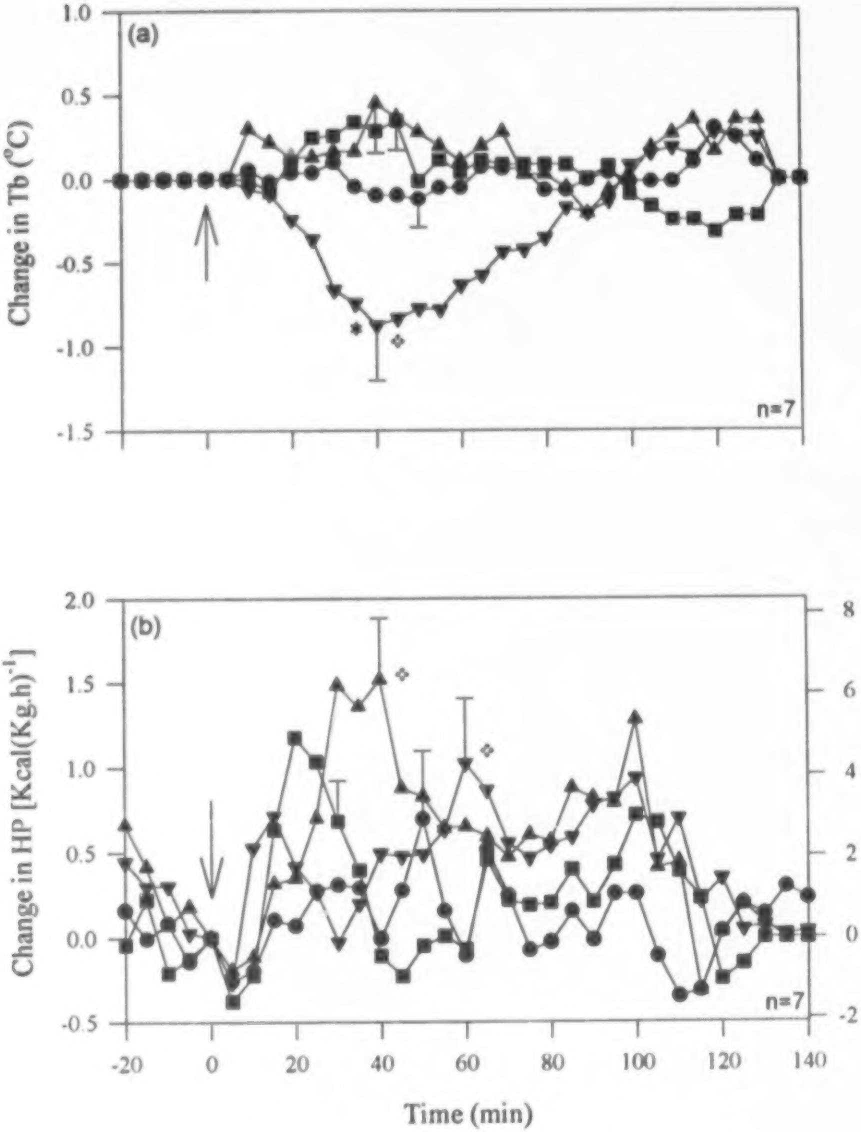


Figure III.7 Thermoregulatory effect of hippocampal injection of D-Ala^2-Met-enkephalinamide in non-hibernating ground squirrles at 5°C. (Arrow: injection; closed circle: CSF; closed square: 3.52 n mol ENK; closed triangle: 17.58 n mol ENK; closed reversed triangle: 87.92 n mol ENK). * significantly different from its pre-injection level; ❖ significantly different from the CSF treatment.

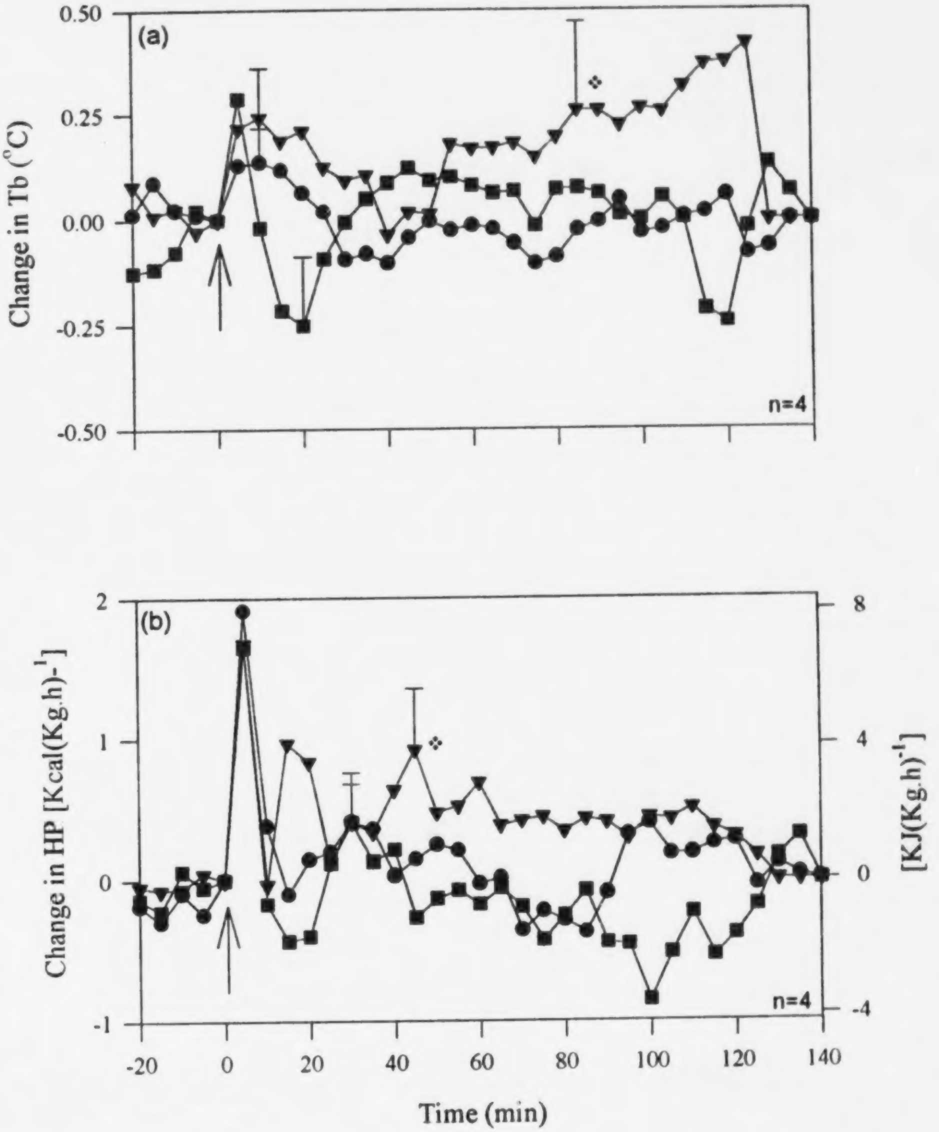


Figure III.8 Thermoregulatory effect following hippocampal application of D-Ala^{2}-Met-enkephalinamide in the hibernating phase at 5°C. (Arrow: injection; closed circle: CSF; closed square: 3.52 n mol ENK; closed reversed triangle: 87.92 n mol ENK). ❖ significantly different from the vehicle (CSF) treatment.

different from the vehicle injection ($F_{1,198}$=9.79, $P<0.01$, two-way ANOVA).

In contrast to met-enkephalinamide, U50488 did not cause any consistent or significant thermoregulatory response at doses up to 108.33 n mol when injected into the hippocampal region of non-hibernating (Table III.1) and two hibernating (data not shown) ground squirrels.

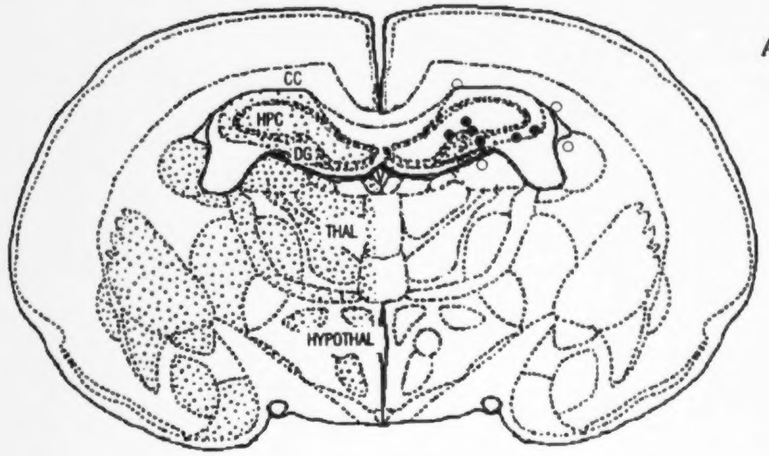
The microinjection sites are shown in Figure III.9. Met-enkephalinamide caused a biphasic hyperthermia/hypothermia thermoregulatory response in the non-hibernating ground squirrels only when injected into the CA2/CA3 and dentate gyrus of the hippocampal region (closed circles). However, in any other surrounding areas (open circles), enkephalinamide did not elicit significant thermoregulatory effect and therefore were not included in evaluating the thermoregulatory responses.

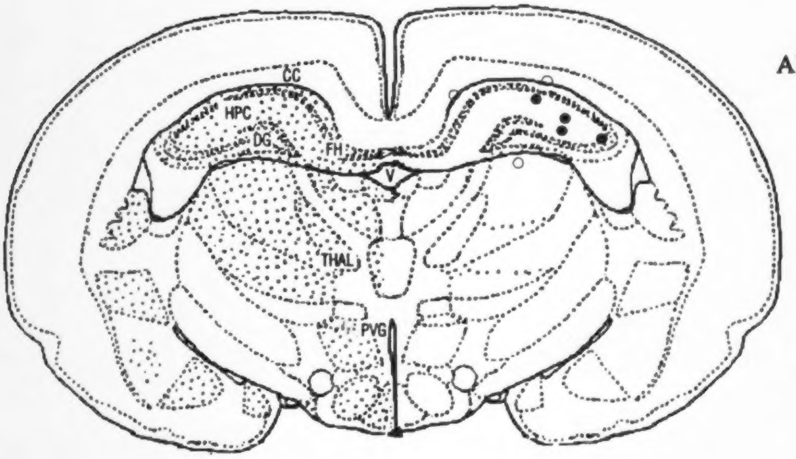
Effect of 5-HT in thermoregulation in the hippocampus

Intra-hippocampal injection of 5-HT even at 51.63 n mol did not alter either the Tb or the HP of non-hibernating Columbian ground squirrels (Figure III.10). Similarly, when applied to the hippocampus of ground squirrels in the hibernating phase, 5-HT did not elicit any significant thermoregulatory response either (Figure III.10).

Discussion

Maintenance of a relatively constant Tb is one of the important features of mammalian homeostasis. The POAH has been known for decades to be the primary thermoregulatory "controller". The POAH receives and integrates central and peripheral thermal stimuli to activate appropriate effectors (Alder et al. 1988, Boulant et al. 1989, Schonbaum and Lomax 1990). Peripheral cold


AP=11mm
CC
HPC
DG
THAL
HYPOTHAL


AP=10 mm
CC
HPC
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V
THAL
PVG

Figure III.9 Stereotaxic map of met-enkephalinamide injection loci in the hippocampal region of Columbian ground squirrles. (closed circles indicate the sites at which ENK induced a biphasic hyperthermia/hypothermia thermoregulatory response whereas open circles represent the loci which brought no consistent change in Tb; CC: Corpus callosum; DG: Dentate gurus; FH: Hippocampal fissure; HPC: Hippocampus; HYPOTHAL: Hypothalamus; PVG: Periventricular grey; THAL:Thalamus.)

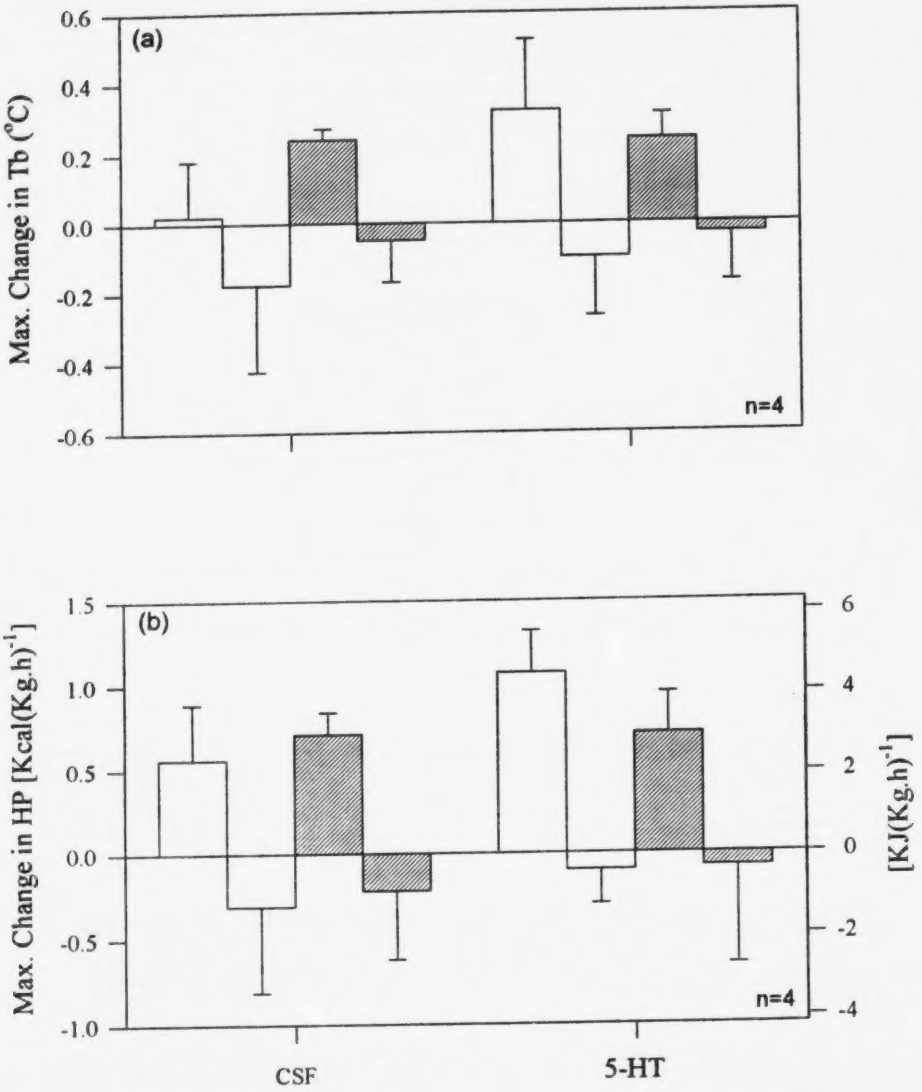


Figure III.10 Maximal increase and decrease in Tb and HP following hippocampal administration of 5-HT (51.63 n mol) in Columbain ground squirrels in the non-hibernating phase (clear bar) and hibernating phase (hatched bar) at 5°C.

stimulus increases the firing of cold-sensitive neurons in the POAH and elicits HP, whereas activation of-warm-sensitive POAH neurons by peripheral warmth causes an increase in heat loss (HL, Boulant and Dean 1986, Boulant et al. 1989). Thus, in most circumstances, Tb is normally regulated within a narrow temperature range by proper adjustment of HP and HL. On the other hand, other CNS regions, such as the posterior hypothalamus, septum, brainstem reticular formation, spinal cord (Boulant et al. 1989, Clark and Lipton 1991) and the hippocampus (Murakami et al. 1984), are also thought to be important in integrating and regulating Tb. This is supported by results of the present study on potential function of opioids and/or 5-HT on the thermoregulatory functions in the septum and hippocampus.

Alteration of Tb by administration of drugs can be either due to changing of set-point or modification of thermoregulatory function (Adler et al. 1988, Clark and Lipton 1991). A change in set-point is characterized by a coordinated adjustment of HP and HL and a consistent shift in Tb in the same direction at both high and low Ta. In contrast, modified thermoregulatory ability is distinguished as a paradoxical change in HP, HL and/or inappropriate response to thermal stimulus. The 5-HT induced decline in Tb, in the present study, appeared to be the result of suppressed HP as the decrease in HP always proceed a drop in Tb (Figure II.1, 2). However, as the decrease in HP was not large enough to account for the full decline in Tb, an increase in HL must have also contributed to the decrease in Tb; the latter was also supported by the observation of the animal's posture (spreading out) following 5-HT injection. These results are in concordance with previous experiments that 5-HT excites warm-sensitive neurons and inhibits cold-sensitive neurons (Hori and Nakayama 1973, Jell 1974). However, the effects of 5-HT on cold- and/or warm-sensitive neurons vary with the CNS regions investigated (Myers and Lee 1989). The exact mechanism of the present observed thermoregulatory response following septal injection of 5-HT needs to be further verified by behavioral studies and single cell recording.

Although the change in Tb after 5-HT injection in the septum at 5°C was similar to that at 20°C, the fall in Tb at 5°C was larger than that of the same dose at 20°C. Since at 20°C, the cold-sensitive neurons of the animal were only minimally activated and the resting HP was relatively low, the decrease in regulatory HP by 5-HT was necessarily low (Figure III.1). At 5°C, cold stimulated the cold-sensitive neurons and induced a greater elevation of regulatory HP than at 20°C. Thus, at 5°C, 5-HT could suppress HP to a greater extent than at 20°C (Figure III.2). In addition, since the HL at 5°C was also higher than that at 20°C, an enhancement of HL by 5-HT would further contribute to the greater decrease in Tb at 5°C.

Interestingly, the hypothermic effect of 5-HT injected into the lateral septum was similar to that after high dose of met-enkephalinamide applied to the same region (Lee et al. 1989). It has been suggested, however, that the hypothermic response of 5-HT administration in the septum is not mediated by activation of the opioid system in rats (Cui et al. 1993). It is, thus, possible that the reverse may be true: opioids modulate the serotonergic system in the lateral septum to elicit the hypothermic effect. This seems to be the case as (1) the initiation of Tb decrease after 5-HT administration was always sooner than that of met-enkephalinamide; and (2) met-enkephalinamide-induced hypothermia could be attenuated by pre-treatment with 5-HT antagonist cyprohaptidine, although cyprohaptidine alone had no significant thermoregulatory effect (Figure III.5). Therefore, septal administration of met-enkephalinamide leads to activation of the serotonergic system which in turn induces the hypothermic response as seen in the non-hibernating ground squirrels. This interpretation is further supported by previous work that hypothermia induced by peripheral or central administrated morphine was mediated by the serotonergic pathways (Oka 1978, Burks and Rosenfeld 1979).

Similar to previous observations involving the lateral septum (Lee et. el. 1989), intra-hippocampal injection of D-Ala^2-Met-enkephalinamide also induced a biphasic thermoregulatory responses in the non-hibernating ground squirrels. Low doses (3.52 and 17.58 n mol) of D-Ala^2-Met-enkephalinamide caused hyperthermia, while high dose (87.92 n mol) produced hypothermia at Ta of 20°C. The hyperthermia provoked by low doses of met-enkephalinamide was due in part, by an increase in HP (Figure III.6). However, at Ta=5°C, the cold-sensitive neurons had been activated for HP before drug application and further enhancement of the neuronal HP activity by the drug may not be noticeable or feasible. Therefore, the hyperthermic response provoked by low doses of met-enkephalinamide diminished at 5°C. Furthermore, the HL at 5°C was much higher than at 20°C. The outcome of both an increase in HL due to the cold and a minimum augmentation of HP appeared to be responsible for the attenuated hyperthermic effect at low doses and enhancement of hypothermic effect at high dose of met-enkephalinamide at 5°C. In contrast to the met-enkephalinamide elicited hypothermia in the lateral septum (Lee et al. 1989), the hypothermic effect following hippocampal administration of met-enkephalinamide was not a result of suppression in HP (Figure III.6, 7). It appeared to be solely due to an elevation in HL. In addition, 5-HT administration in the hippocampus of Columbian ground squirrels did not evoke significant thermoregulatory responses. Therefore, the enkephalinamide-induced hypothermia in the hippocampus differs from that of the lateral septum, and may be mediated by other mechanisms.

Since D-Ala^2-met-enkephalinamide binds to δ receptors and with some potency to μ receptors, its biphasic effects in thermoregulation could not exclude the μ component (hyperthermia) as seen after icv administration of μ agonist DAGO alone (Spencer et al. 1988, 1990, Handler et al. 1992). The thermoregulatory outcome of met-enkephalinamide, thus, depends on the ratio and affinity of δ and μ receptors in the hippocampal or lateral septal regions. Although μ agonists also produce a hypothermic response under thermal or environmental stress in rats (Spencer et al. 1988, Handler et al. 1992), it is generally agreed that μ agonists mainly cause hyperthermia in rodents (Adler et al. 1988, Shukla and Dhawan 1988, Burks 1991). The possible role of δ receptor in thermoregulation is still controversial since either a lowering or no change in the set-point by δ agonists in rats have been reported (Spencer et al. 1990, Burks 1991, Geller et al. 1991, Broccardo and Improta 1992, Handler et al. 1992, 1994). It is speculated from the present study that activation of δ receptors induces hypothermia but this is somewhat "diluted" by the hyperthermic μ effects at low doses of met-enkephalinamide. This view is supported by our microdialysis studies in the lateral septum (chapter two); activation of the δ receptor stimulated 5-HT release which in turn induced hypothermia. However, in the hippocampus, further thermoregulatory studies with specific δ receptor agonists and/or μ antagonists need to be done before any conclusion can be drawn.

Another controversial aspect of opioid and thermoregulation centers is on the potential function of the κ receptors (Adler et al. 1988, Burks 1991). The present study demonstrated that k agonist, U50488 up to a dose of 108.33 n mol, did not produce any profound thermoregulatory effect following administration into the lateral septum or hippocampus of non-hibernating Columbian ground squirrels. However, intra-peritoneal injection of U50488 (0.11 mmol/Kg weight) produced a long-lasting (5 to 6 hours) and severe hypothermia (almost 7°C decrease) in the non-hibernating ground squirrels (unpublished observation). This supports the contention that κ-mediated hypothermia is largely due to activation of peripheral rather than central κ receptors (Adler et al. 1988, Adler et al. 1991).

The other important finding of the present study was the reduction in the ability of met-enkephalinamide and 5-HT to modulate thermoregulation in the hibernating phase. The right-hand shift in enkephalinamide thermoregulatory dose-response curves observed in the hibernating phase (Figure III.3, 8) suggests desensitization or down-regulation of opioid receptors in hibernation. This agrees with previous studies (Wang et el. 1987, Lee et al. 1989) on seasonal differences of thermoregulatory response following icv and septal application of morphine and met-enkephalinamide in hibernators. However, rather than an enhanced hypothermia following 5-HT administration, when injected icv as reported by Glass and Wang (1979), the efficacy of 5-HT induced hypothermia by septal administration was lower in the hibernating phase. This may be due to the difference in injection locations and, thus, activating different pathways. This speculation is supported by the observations of opposite or attenuated effects on HP following 5-HT injection in the lateral septum and icv of the non-hibernating ground squirrels (Figure III.3, Glass and Wang 1979). Because the drug delivered by icv could diffuse out to the surrounding regions, its effect would be a composite of all affected effectors.

Based on previous and present experiments, δ opioid induces 5-HT release (Chapter Two) and an increase in endogenous opioid activity (demonstrated by down-regulation of opioid receptors) appears evident prior to or during the hibernating phase (Beckman et al. 1986, Wang et al. 1987, Lee et al. 1989). It is possible that an increase in 5-HT activity may then also be expected in the hibernating phase as has been demonstrated previously (Popova and Voitenko 1981). Long term increase in serotonergic activity, therefore, may result in down-regulation of serotonin receptors (Gothert 1991). The result of the decrease in responsiveness to 5-HT will lead to the attenuation of 5-HT hypothermia and other inhibitory influences on the septo-hippocampal complex (Chapter One) and, thus, may facilitate arousal.

References

Adler M.W., Geller E.B., Rosow C.E., and Cochin J. (1988) The opioid system and temperature regulation. Ann Rev Pharmacol Toxicol 28:429-49

Adler M.W., Bradley E., Martinez R., and Geller E.B. (1991) Production of hypothermia in the guinea pig by a kappa-agonist opioid alone and in combination with chlorpromazine. Pharmacol Biochem Behav 40:129-132

Adler M.W. and Geller E.B. (1992) Physiological functions of opioids: temperature regulation. Handbook of experimental pharmacology. Vol. 104/II Opioids II Herz A., Akil H., and Simon E.J. (eds), pp 205-238, Pergamon Press, New York.

Beckman A., Dean R. R.,and Wamsley J. K. (1986) Hippocampal and cortical opioid receptor binding: changes related to the hibernation state. Brain Res 386:223-231

Bligh J. (1979) The central neurology of mammalian thermoregulation. Neuroscience 4:1212-1236

Boulant J.A. and Dean J.B. (1986) Temperature receptors in the central nervous system. Ann Rev Physiol 48:639-654

Boulant J.A., Curras M.C., and Dean J.B. (1989) Neurophysiological Aspects of thermoregulation. Advances in comparative and environmental physiology Vol.4 Wang L.C.H. (ed), pp 117-160, Springer-Verlag, Berlin.

Broccardo M. and Improta G. (1992) Hypothermic effect of D-Ala-deltorphin II, a selective opioid receptor agonist. Neurosci Lett 139:209-212

Burks T.F. and Rosenfeld G.C. (1979) Neurotransmitter mediation of morphine hypothermia in rats. Life Sci 24:1067-1074

Burks T.F. (1991) Opioids and opioid receptors in thermoregulation. Thermoregulation: Pathology, Pharmacology and Therapy. Schonbaum E. and Lomax P. (eds), pp 489-508, Pergamon Press, New York.

Chopin P., Moret C., and Briley M. (1994) Neuropharmacology of 5-hydroxytrypamine$_{1B/D}$ receptor ligands. Pharmac Ther 62:385-405

Clark W.G. (1983) Effects of opioid peptides on thermoregulation. Fed Proc 40:2754-2759

Clark W.G. and Lipton J.M. (1983) Brain and pituitary peptides in thermoregulation. Pharmac Ther 22:249-297

Clark W.G. and Lipton J.M. (1991) Brain and pituitary peptides in thermoregulation. Thermoregulation: Pathology, Pharmacology and Therapy. Schonbaum E. and Lomax P. (eds), pp 509-560, Pergamon Press, New York.

Cui Y., Lee T.F., and Wang L.C.H. (1993) Thermoregulatory responses following injection of 5-hydroxytryptamine into the septohippocampal complex in rats. Pharmacol Biochem Behav 45:935-939

Disturnal J.E., Veale W.L., and Pittman Q.J. (1986) The ventral septal area: electrophysiological evidence for putative arginine vasopressin projections onto thermoresponsive neurons. Neuroscience 19:795-802

Florant G.L. and Heller H.C (1977) CNS regulation of body temperature in euthermic and hibernating marmots. Am J Physiol 232:R203-R208

Florant G.L, Turner B.M, and Heller H.C. (1978) Temperature regulation during wakefulness, sleep, and hibernation in marmots. Am J Physiol 235:R82-R88

Geller E.B., Piliero T., and Adler M.W. (1991) Lack of evidence for a role of delta receptor in temperature regulation in the rat. FASEB J 4:A881

Glass J.D. and Wang L.C.H. (1979) Thermoregulatory effects of intracerebroventricular injection of serotonin and a monoamine oxidase inhibitor in a hiberantor, *Spermophilus richardsonii.* I Thermal Biol 4:149-156

Gothert M. (1991) Presynaptic effects of 5-HT. LTP, Galanin, Opioids, autonomic and 5-HT Stone T.W. (ed), pp 314-329, Taylor & Francis, London.

Handler C.M., Geller E.B., and Adler M.W. (1992) Effect of m-, k-, and d-selective opioid agonists on thermoragulation in the rat. Pharmacol Biochem & Behav 43:1209-1216

Handler C.M., Piliero T.C., Geller E.B., and Adler M.W. (1994) Effect of ambient temperature on the ability of *mu-*, *kappa-*, and *delta-*seeltive opioid agonists to modulate thermoregulatory mechanisms in the rat. J Pharmacol Exp Ther 268:847-855

Haubrich D.R. and Blake D.W. (1971) Modification of the hypthermic action of morphine after deplation of brain serotonin and catecholamines. Life Sci 10:175-180

Hori T. and Nakashima T. (1973) Effects of biogenic amines on central thermoresponsive neurons in the rabbits. J Physiol 232:71-85

Hori T., Kiyohara T., and Nakashima T. (1989) Thermosensitivie neurons in the brain - the role in homeostatic function. Thermal Physiology. Mercer J.B. (ed), pp 3-12, Excerpta Medica, Amsterdam.

Jansky L. (1990) Neuropeptides and the central regulation of body temperature during fever and hibernation. J Therm Biol 15:329-347

Jell R.M. (1974) Responses of rostral hypothalamic neruons to peripheral temperature and to amines. J Physiol 240:295-307

Kiyohara T., Hori T., Shibata M., Nakashima T., and Osaka T. (1984) Neural inputs to preoptic thermosensitive neurons - histological and electrophysiological mapping of central connections. J Therm Biol 9:21-26

Kleiber M (1975) The Fire of Life: An Introduction to Animal Energetics pp 124-128, Wiley, New York.

Komiskey H.L. and Rudy T.A. (1977) Serotonergic influences on brain stem thermoregulatory mechanisms in the cat. Brain Res 134:297-315

Lee T.F., Nurnberger F., Jourdan M.L., and Wang L.C.H. (1989) Possible involvement of septum in seasonal changes in thermoregulatory responses to met-enkephalinamideamide in ground squirrels. Thermoregulation: Research and Clinical Applications Lomax P. and Schonbaum (eds), pp 200-203, Pergamon Press, New York.

Murakami N., Morimoto A., and Watanabe T. (1984) Radioactive deoxyglucose uptake in the rat CNS during thermoregulatory responses. Thermal Physiology Hales J.R.S. (ed), pp 105-108, Raven Press, New York.

Myers R.D. and Lee T.F. (1989) Neurochemical aspects of thermoregulation. Advences in comparative and enviromental physiology Vol.4 Wang L.C.H. (ed), pp 161-203, Springer-Verlag, Berlin.

Nakashima T., Hori T., Kiyohara T. (1989) Intracellular recording analysis of septal thermosensistive neurons in the rat. Thermal Physology Mercer J.B. (ed), pp 69-72, Excerpta Medica, Amsterdam.

Oka T. (1977) Role of 5-hydroxytryptamine in morphine-, pethidine-, and methadone-induced hypothermia in rats at low ambient and room tempperature. Br J Pharmac 60:323-330

Oka T. (1978) 5-HT and narcotic-induced hypothermia. Gen Pharmacol 9:151-154

Osaka T., Hori T., Kiyohara T., Shibata M., and Nakashima T. (1984) Changes in body temperature and thermosensitivity of preoptic and septal nerons during huppocampal stimulation. Brain Res Bull 13:93-98

Popov N.K. and Voitenko N.N. (1981) Brain serotonin metabolism in hibernation. Pharmacol Biochem & Behav 14:773-777

Samanin R.K. and Garattini S. (1972) Abolition of the morphine effect on body temperature in midbrain raphe lesiones rats. J Pharm Pharmac 24:374-377

Schonbaum E. and Lomax P. (1990) Temperature regulation and drugs: an introduction. Thermoregulation Physiology and Biochemistry Schonbaum E. and Lomax P. (eds), pp 1-16, Pergamon Press, New York.

Shukla R. and Dhawan B.N. (1988) Peptides and thermoregulation. Opioid peptides: an update: DINA research monograph 87 Rapaka R.S. and Dhawan B.N. (eds), pp 192-208, DINA publication, Rockville.

Spencer R.L., Hruby V.J, and Burks T.F. (1988) Body temperature response profiles for selective mu, delta, and kappa opioid agonists in restrained and unrestrained rats. K Pharmacol Exp Ther 246:92-101

Spencer R.L., Hruby V.J, and Burks T.F. (1990) Alteration of thermoregulatory set point with opioid agonists. J Pharmacol Exp Ther 252:696-705

Wang L.C.H. (1978) Factors limiting maximum cold-induced heat production. Life Sci 23:2089-2098

Wang L.C.H., Lee T.F. and Jourdan M.L. (1987) Seasonal difference in thermoregualtory responses to opiates in a mammalian hibernator. Pharmacol Biochem Behav 26:565-571

Zeisberger E. (1990) The role of septal peptides in thermoregulation and fever. Thermoreception and Temperature Regulation. Bligh J. and Voigt K. (eds), pp 273-283, Springer-Verlag, Berlin.

CHAPTER FOUR

Autoradiographic determination of changes in opioid receptor binding in the hibernation cycle

Introduction

Since the discovery of opioid receptors in the central nervous system (CNS) in the 70's (Pert and Snyder 1973, Simon et al. 1973, Terenius 1973), their heterogeneity and distribution have been studied extensively (for reviews see Akil et al 1988, Chang 1984, Khachaturian et al. 1985). Based on ligand binding, biological assays, and behaviour effects (Gilbert and Martin 1976, Martin et al. 1976, Lord et al. 1977, Zukin and Zukin 1981), mainly three types of opioid receptors, μ, δ, and κ, have been thoroughly investigated and well defined (Simon 1991, Loh and Smith 1990, Ueda et al. 1991). The μ selective agonists are D-Ala^2,N-Me-Phe^4,Gly-ol^5]enkephalin (DAGO and [N-MePhe^3,D-Pro^4]morphiceptin (PL017). [D-Pen^2,D-Pen^5]enkephalin (DPDPE),-[D-Ser^2,Leu^5]enkephalin (DSLET), and [D-Ala^2,D-Lue^5]enkephalin (DADLE) are the selective agonists for δ receptors. The selective agonists for κ receptors are trans-(dl)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate (U50488), ethylketocyclazocine (EKC), and dynorphin A. All three types of opioid receptors have been illustrated existing in the CNS but with different distribution patterns and densities (Atweh and Kuhar 1983, Mansour et al. 1988) and they vary among species (Robson et al. 1985, Herkenham and McLean 1988, Sharif and Hughes 1989). Activation of different receptors by various selective ligands is responsible for the diverse pharmacological phenomena of opioids.

One possible physiological role of opioids is their involvement in mammalian hibernation. Experimental evidence has suggested that an increase in opioid activity is essential for the entry and maintenance of hibernation (see review Wang 1993). Changes in endogenous opioid activity can be a result of changes in the concentration of endogenous opioid peptides and/or spontaneous changes in the receptor number or conformation or altered gene expression of opioid receptors. Spontaneous changes in opioid receptors have been demonstrated to correlate with specific pathological and developmental events (Barg and Simantov 1991, McConnaughey et al. 1992). On the other hand, chronic increases in opioid concentration, either endogenously or administrated exogenously, could induce desensitization or down-regulation of opioid receptors (Bhargava et al. 1991, Tao et al. 1991, Stein et al. 1992). This desensitization or down-regulation has been demonstrated to be responsible for the decrease in opioid receptor sensitivity to exogenous opioids (Dingledine et al. 1983). Therefore, previous observations that a decrease in opioid receptor responsiveness in mediating thermoregulation and modulating the release of other neurotransmitters in certain brain regions during hibernation may be a result of increased endogenous opioid activity (Lee et al. 1989, Kramarova et al. 1991, Cui et al. 1993). The present study is, thus, aimed at directly and quantitatively determining the changes in opioid receptors in different states of a hibernation cycle.

Receptor binding and receptor autoradiography are two common methods used to directly evaluate the receptor density of a specific brain region and may correlate with the observed changes in a specific physiological or pathological event. However, there are some limitations to receptor binding assays. For instance, because a relatively large amount of homogeneous sample is required for one complete assay, for smaller brain regions, pooled samples from several animals have to be used. However, in the present study, it is almost impossible to obtain two hibernating animals of identical state to pool. So, the receptor binding approach is not feasible. In addition, if receptor density only changes in one nucleus of a large region, this change may be masked by other unaltered nuclei of the same region when receptor binding is employed. Finally, if a change is indeed demonstrable by the receptor binding assay, it is still unable to pin-point the exact nucleus (or nuclei) of the change. With the improvement in instrumentation, it is now possible to quantitatively determine the receptor density in a nucleus and compare its change under different physiological conditions using receptor autoradiography. Therefore, receptor autoradiography has been chosen for this study.

Although a decrease in opioid receptor binding with dihydromorphine has been illustrated in the hippocampus and cortex of hibernating animals (Beckman et al. 1986), this study mainly provided information on changes in μ receptors. On the other hand, a reduction in κ, but not μ or δ, receptor binding in the brain homogenate of hibernating ground squirrel (Aloia et al. 1985) and no change in either affinity or number of opioid receptors in the hypothalamus and hippocampus of hibernating ground squirrels (Kolaeva et al. 1989) have also been reported. These isolated, seemingly conflicting observations did not provide a holistic vision on changes of opioid activity during hibernation. Because of the complexity of multi-opioid receptors, it is not necessarily that all three types of receptor change, or even if so, change in unison during hibernation. It is, therefore, apparent that a systematic investigation on multi-opioid receptor activities throughout a hibernation cycle would further clarify the regulatory role of opioids in hibernation. The limbic opioid activity was selected as the focus of the present study because of the hypothesized crucial inhibitory role of the limbic system in regulating the onset and/or maintenance of hibernation (Heller 1979, Beckman and Stanton 1982, Belousov 1993, and also see Chapter One).

Methods

Animals The experimental protocols were approved by the University of Alberta Animal Use Committee, following the guidelines of the Canadian Council on Animal Care. Both male and female Columbian ground squirrels (*Spermophilus columbianus*) were trapped in the foothills of the Rocky Mountains in Alberta. They were kept individually at 22°C under 12L:12D photoperiod and *ad lib.* food (rodent chow, mixed grains, and sunflower seeds) and water. The body weights of the squirrels were recorded weekly and monitored throughout the year. The onset of the hibernation season in an individual was characterized by its rapid weight gain followed by a plateau. When this occurred, the animals were transferred to a cold (4±1°C) and dark walk-in environmental chamber to facilitate the transition to hibernation. The activities of the animals were then checked and recorded daily. The hibernating state of the animal was characterized by a lack of movement, slow and regular breathing (less than one per minute), and the remaining of sawdust on its back when placed the previous day. Hibernating animals (body temperature, Tb=5-7°C) were used only after they had completed at least two hibernation bouts and on the second day of the current hibernation bout. The non-hibernating animals (Tb=37-38°C) were the active ground squirrels with relatively stable body weight for at least two months.

Preparation of brain sections Non-hibernating or hibernating Columbian ground squirrels were terminated by decapitation. The brains were removed from the skull rapidly and immediately frozen in powdered dry ice. They were then sectioned coronally at 10 μm using a cryostat microtome at -17°C. The sections were mounted on pre-treated (over night acid wash and gelatinized) microscope slides and dried at 20°C for 2 hours. The brain slices were stored in refrigerator (4°C) for no longer than a week before use.

Determination of optimal incubation conditions for the binding of three types of opioid receptor

The initial incubation conditions for the opioid ligands were selected based on previous reports (Wood 1986). The brain sections were incubated with tritiated ligands at the concentration around the reported K_d value and the incubation time was set according to that normally used in the literature. The total binding and non-specific binding were determined using liquid scintillation counting and specific binding was obtained by subtracting non-specific binding from the total binding.

Washout curve For total μ receptor binding, brain slices were incubated in 2 nM of [^{3}H]DAGO with 50 mM phosphate buffer (pH=7.4) containing 0.2% BSA and enkephalinase inhibitors (50 mM bacterin, 20 μM bestatin, and 2 mM captopril) for 120 minutes at 20°C; 2 nM [^{3}H]DAGO in the presence of 2 μM DAGO was used for non-specific binding. The slices were then washed in ice-cold phosphate buffer for various periods of time (4 rinses, 2 seconds to 10 minutes each time) with a final in-out dip in ice cold distilled water. The brain sections were wiped off with GF/C glass microfibre filter and the radioactivity of the slices was counted by liquid scintillation spectrometry (Beckman LS 6500). For δ binding, brain sections were incubated with 4 nM [^{3}H]DPDPE for 60 minutes at 20°C. The non-specific binding was evaluated by incubating brain sections in 4 nM [^{3}H]DPDPE with 4 μM DADLE. The slices were also washed in ice-cold phosphate buffer for four times, 2 seconds to 10 minutes each time, and the radioactivity measured thereafter. For κ binding, since the κ receptor has been reported as unstable at high incubation temperature (Wood 1986), all incubations for κ binding were performed at 4°C. In addition, as EKC binds κ receptor specifically only when μ and δ binding sites are occupied, the total binding was determined using 3 nM of [^{3}H]EKC in the presence of 100 nM DAGO and 100 nM DADLE with a incubation time of 90 minutes. For non-specific binding, brain sections were incubated with [^{3}H]EKC in the presence of 3 μM bremazocine, 100 nM DAGO, and 100 nM DADLE. The wash out time was similar to that used for μ and δ receptor binding. The radioactivity of brain sections was then plotted against the washing time for all three types of opioid receptor.

Pre-incubation curve Brain sections were first incubated in 50 mM phosphate buffer for various periods of time, from 0 to 40 minutes, at room temperature for μ and δ binding and 4 °C for κ binding before incubating with tritiated ligands. They were then blow-dried at room temperature for 10 minutes and incubated with corresponding tritiated ligand under the same condition as described in the above section. The incubation was terminated by washing the slices in ice-cold phosphate buffer for a specific time period determined from the previous section based on a high ratio of specific/non-specific binding. Radioactivity of the brain sections was detected using liquid scintillation counting and the radioactivity was plotted against pre-incubation time.

Association curve Based on the pre-incubation curve, brain slices were pre-incubated for a particular time period which provided the best specific binding and the ratio of total/non-specific binding. The slices were then dried and incubated with corresponding radioactive ligands with the above used conditions for different durations (from 0 to 180 minutes) before washing with ice-cold phosphate buffer. The radioactivity of each slice was also measured and plotted against the incubation time.

Saturation curve The slices were pre-incubated and then incubated with radioactive μ, δ, or κ ligands at various concentrations under optimal conditions as determined in the above experiments. The total binding, non-specific binding, specific binding, and bound/free of tritiated ligand at different concentrations were evaluated and calculated. Saturation curves were constructed using radioactivity of the slices vs. the concentration of the ligand. The Scatchard plot was obtained by plotting bound/free of the ligand vs. bound ligand. The K_d and B_{max} values of each ligand in the brain of ground squirrels were determined from the Scatchard plot (Wood 1986).

Displacement The brain sections were incubated with radioactive μ, δ, or κ

ligands in the presence of various μ, δ, and κ opioid agonists, antagonists, or non-opioid ligands. The specific binding in each condition was determined and expressed as the percentage of the specific binding in the absence of any cold ligand. The specificity of each ligand to a certain receptor type was represented by IC_{50} (inhibition constants) which is defined as the concentration of the cold ligand at which it displaces 50% of the specific binding of tritiated ligand..

Preparation of standard brain paste To quantify the opioid receptor density bound by tritium-labelled ligands, homemade brain paste was used. The whole brain (including forebrain, midbrain, cerebellum, and medulla) of a Colombian ground squirrel was homogenized with PYREX (No. 7725) homogenizer at 0 °C. It was degassed in a 12 X 75 mm test tube with centrivap concentrator (LABCONCO) and frozen in dry ice. The brain paste was removed from the test tube, sectioned at a thickness of 10 μm at -17°C and mounted on gelatinized microscope slide. At the same time, the dry weight and protein content (Bradford 1976) of ten brain paste sections were determined. Because of the abundance of GABA receptors in the brain, each of the mounted brain paste section was incubated with different amount of [^{3}H]GABA to obtain evenly labelled standard paste sections. Following 120 minutes incubation at 20°C, the brain paste sections were blow-dried and finally washed in ice-cold distilled water for two seconds before air dried. They were used with brain sections in the development of autoradiogram of opioid receptor binding. The radioactivity of each brain paste section was subsequently measured after the development of autoradiogram to construct a standard curve.

Autoradiography Brain sections of non-hibernating and hibernating ground squirrels were pre-incubated, incubated, and washed in pairs at the optimum conditions for each of the three types of opioid receptors elucidated from the above experiments. After the final wash, the slices were immediately dried by blowing cool, dry air onto them. These slices along with the [^{3}H]GABA labelled standard brain paste were then mounted in Kodak X-ray cassettes and the tritium hyperfilms (Amersham) were put against them tightly. They were kept at -70°C for 10 to 20 weeks. The exposed hyperfilms were processed with Kodak developer D-19 for 5 minutes at 20°C and rinsed in water for 30 seconds before being fixed in rapid fixer for 5 minutes. The films were finally rinsed in running water for 20 minutes and air dried.

Quantification of autoradiograms The density of three types of opioid receptor binding in various brain regions of non-hibernating and hibernating ground squirrel was analysed using a computer-assisted image analysis system. The image was captured by a video camera NC-68L (Mti Series 68, DAGE-MTI Inc) which was connected to a JENAMED microscope (Carl Zeiss Jena). This system evaluated the optical density of the autoradiogram in a certain pointed region (ranging from 0.0369 mm^2 to 0.922 mm^2) and assigned its average frequency as grey levels (ranging from 0 to 64) using Genias 25 (version 1.3) software (Joyce-Loebl). The optical density of the standard brain paste image, which was co-exposed to the same hyperfilm along with those brain sections, was also determined simultaneously. The optical density of each standard brain paste was plotted against its radioactivity per unit area to construct a best fit standard curve. The radioactivity per unit area in each examined brain region was converted from the optical density using the standard curve. The specific binding of each opioid ligand to the corresponding receptor in a certain brain region was measured five times with five adjacent sections of the same region and averaged after it was corrected for non-specific binding.

Statistics To analyse changes in receptor binding of non-hibernating and hibernating ground squirrels, the receptor binding in each brain region was compared using the Mann-Whitney non-parametric tests because of the small sample pool and non-normality distribution. The significance was set at $P < 0.05$.

Chemicals [^{3}H]DAGO (52.5 Ci/mmol), [^{3}H]DPDPE (33.0 Ci/mmol), and [^{3}H]EKC (18.7 Ci/mmol) were Dupont products. [^{3}H]GABA (99.6 Ci/mmol) and hyperfilms were purchased from Amersham. Nor-BNI (Nor-binaltorphimine dihydrochloride), naltrindole, U50488, and bremazocine were obtained from Research Biochemicals Incorporated. DAGO, DADLE, Naloxone, bacterin, bestatin, and captopil were acquired from Sigma. Other chemicals used in the experiment were obtained from BDH.

Results

Washout curves of brain sections binding with opioid ligands

As shown in Figure IV.1a, after the incubation of brain sections with 2 nM of [^{3}H]DAGO in the presence or absence of 2 μM DAGO for 120 minutes, the total [^{3}H]DAGO binding and non-specific binding decreased drastically within the first minute of wash and then gradually decreased with the increase in wash time. The specific binding of [^{3}H]DAGO decreased slowly throughout the washing period. However, the ratio of total binding versus non-specific binding increased progressively as wash time increased. To achieve both a high specific binding and a good ratio of total binding over non-specific binding, the optimal washout time for [^{3}H]DAGO was determined to be 1 minute for 4 times (a total of 4 minutes).

For δ receptor binding, the brain sections were incubated in 4 nM [^{3}H]DPDPE with or without 4 μM of DADLE for 90 minutes. The κ binding was performed at 4°C, using 3 nM [^{3}H]EKC containing 100 nM DAGO and DADLE in the presence or absence of 3 μM bremazocine for 90 minutes. The pattern of washout curves for both δ (Figure IV.1b) and κ receptor (Figure IV.1c) binding was similar to that of μ receptor. The only difference was that their specific bindings were lower than μ receptor. This is possibly due to the relatively fewer

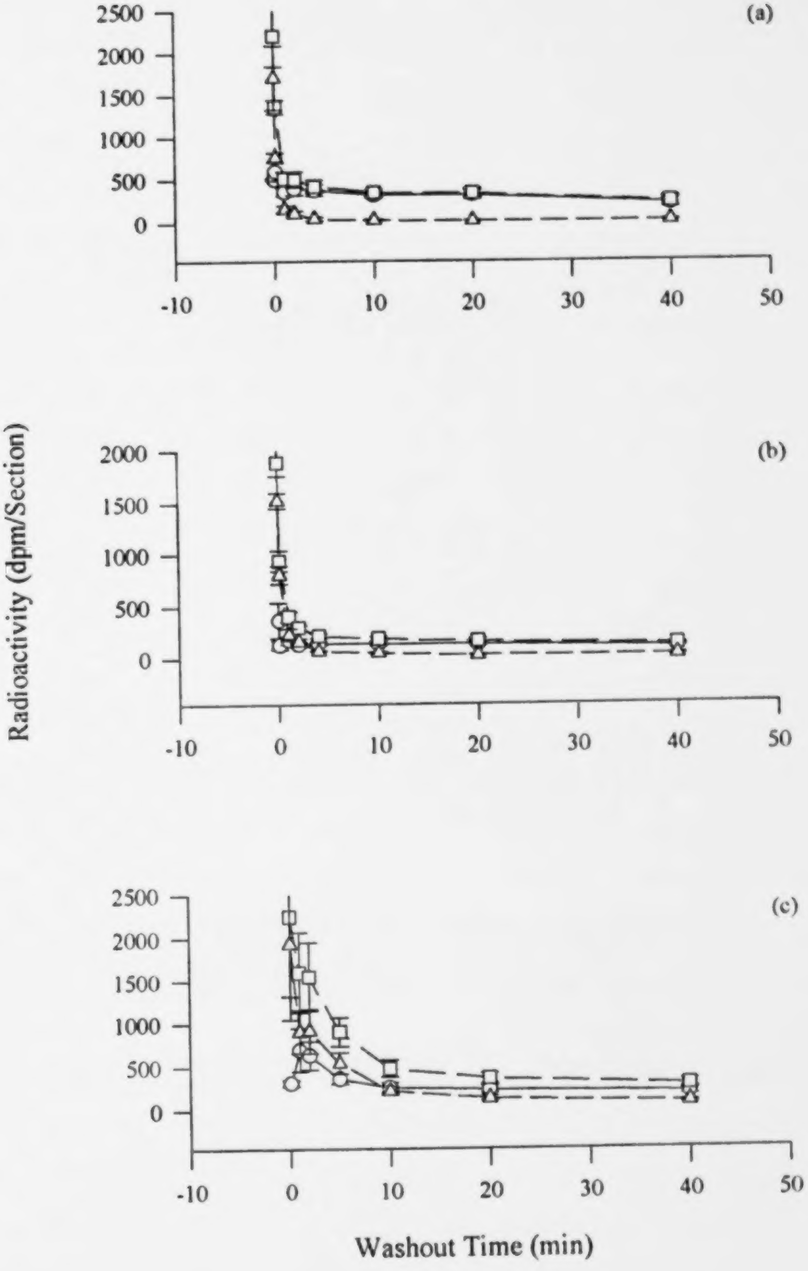


Figure IV.1 Washout curve of [^{3}H]DAGO (a), [^{3}H]DPDPE (b), and [^{3}H]EKC (c) binding in brain sections of Columbian ground squirrel. (open square represents total binding, open circle indicates specific binding, and open triangle denotes non-specific binding, n=3)

δ and κ receptors than μ receptors in the CNS of squirrels (Herkenhman and McLean 1988). According to the above mentioned criteria, the optimal washout time for δ and κ binding was therefore set with 4 X 1 minute (total of 4 minutes) and 4 X 5 minutes (total of 20 minutes), respectively.

Pre-incubation curves

To eliminate the interference of endogenous opioids in receptor binding, the brain slices were pre-incubated first in phosphate buffer to deplete the endogenous peptides. In the first few minutes of pre-incubation, the total binding of [^{3}H]DAGO increased profoundly as the pre-incubation time increased, presumably because of the freeing of opioid receptors from the binding of endogenous opioids (Figure IV.2a). However, with the prolongation of pre-incubation, the total binding of μ receptor started to decrease as the inactivation or instability of opioid receptors increased with pre-incubation time. The non-specific binding of the [^{3}H]DAGO was increased slowly throughout the time course of pre-incubation. Therefore, the specific binding of μ receptor increased within a short period of the pre-incubation (peaked at about minute 5) and then decreased gradually (Figure IV.2a). Similar to the pre-incubation curve of [^{3}H]DAGO binding, the specific binding of [^{3}H]DPDPE and [^{3}H]EKC in the-pre-incubation curve for δ and κ receptors also increased first and then declined slowly (Figure IV.2b and Figure IV.2c). Therefore, the optimal pre-incubation time was 5, 5, and 10 minutes for μ, δ, and κ receptor binding, respectively.

Association curves

To assess how long it takes to reach the equilibrium for each opioid ligand to bind with its receptor, various incubation times were examined. As indicated in Figure IV.3a, b, and c, with the increase in incubation time the total binding of opioid ligands increased rapidly in the early phase of incubation. With further increase in incubation time, the total receptor binding still increased, but, at a slower pace. The non-specific binding increased gradually over the entire

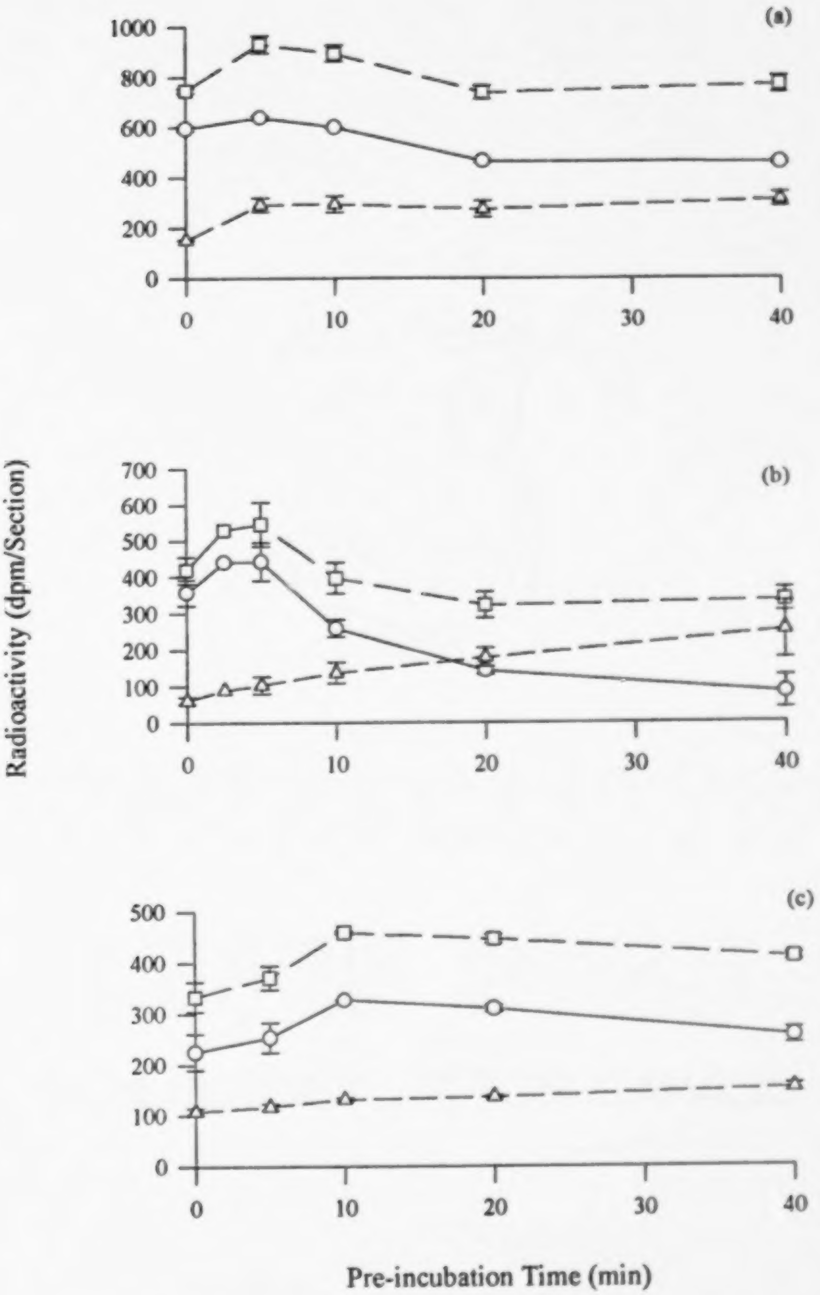


Figure IV.2 Pre-incubation curve of [^{3}H]DAGO (a), [^{3}H]DPDPE (b), and [^{3}H]EKC (c) binding in brain sections of Columbian ground squirrel. (open square means total binding, open circle denotes specific binding, and open triangle is non-specific binding, n=3)

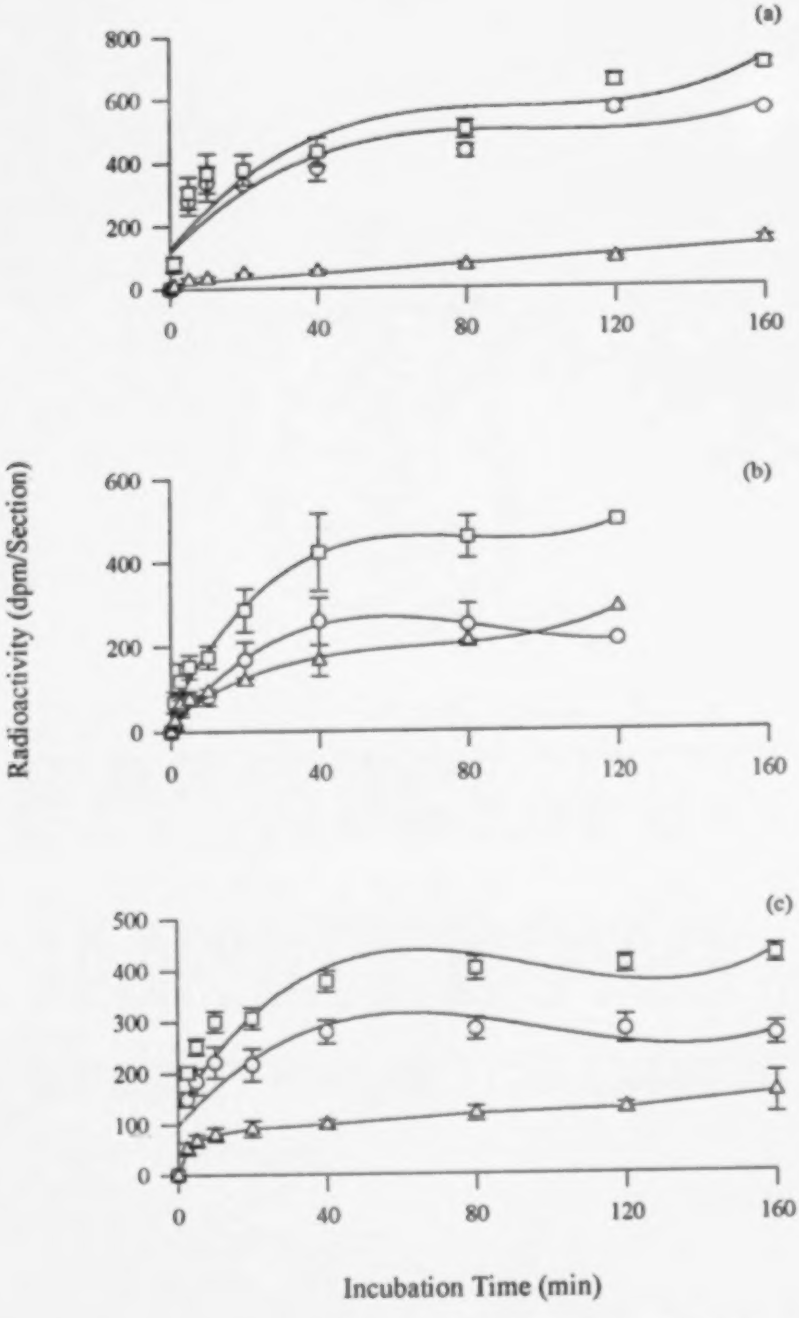


Figure IV.3 Association curve of [^{3}H]DAGO (a), [^{3}H]DPDPE (b), and [^{3}H]EKC (c) binding in brain sections of Columbian ground squirrel. (open square indicates total binding, open circle represents specific binding, and open triangle is non-specific binding, n=3)

incubation time. Therefore, the specific binding of [^{3}H]DAGO to μ receptor increased in the first 120 minutes and levelled off afterwards. For δ and κ receptors, the specific binding reached its maximal level at minute 40 and 80, respectively. Further increase in incubation did not add any more specific binding. Thus, the optimal incubation times for μ, δ, and κ receptor binding used in later experiments were 120, 40, and 80 minutes, respectively.

Saturation curves To appraise the pharmacological characteristics of the opioid receptors in the CNS of ground squirrels, brain sections were incubated with different radioactive ligands in concentrations ranging from 0.125 nM to 19.2 nM using the optimal incubation condition for each type of opioid receptor (Table IV.1). Saturation curves were plotted using total, non-specific, and specific binding vs. concentration of the ligand. As shown in Figure IV.4a, with the increase in concentration of [^{3}H]DAGO, the total binding increased markedly in the low concentration range and slowly in the higher concentration range, while non-specific binding increased gradually throughout the concentration range. Therefore, the specific binding increased rapidly at low concentration and levelled off at high concentration (around 8nM of [^{3}H]DAGO). Using the same set of data, a Scatchard plot was constructed by graphing regression of Bound/Free vs. Bound of [^{3}H]DAGO. The data fell onto a straight line with a r^2 of the regression line equal to 0.95. The X intercept of the Scatchard plot was B_{max}= 325.59 fmol/mg protein in the brain of ground squirrels (Figure IV.4b). The slope of the regression line was $-1/K_d = -0.36$. The K_d value of [^{3}H]DAGO in binding μ receptor of the brain of ground squirrel was, therefore, 2.75 nM. The K_d values of [^{3}H]DPDPE and [^{3}H]EKC obtained from their respective Scatchard plots were 3.70 nM and 3.61 nM, respectively, and the B_{max} of δ and κ receptors in the brain of ground squirrel was 225.70 fmol/mg protein (Figure IV.5b) and 236.75 fmol/mg protein (Figure IV.6b).

Table IV.1 Optimal incubation conditions used in autoradiographic study and the pharmacological characteristics of opioid receptors in the CNS of ground squirrels

	μ receptor	δ receptor	κ receptor
Hot ligands	[^{3}H]DAGO	[^{3}H]DPDPE	[^{3}H]EKC in 100 nM DAGO and DADLE
Concentration	2 nM	4 nM	3 nM
Cold ligands (for non-specific binding)	2 μM DAGO	4 μM DADLE	3 μM Bremazocine, 100 nM DAGO and DADLE
Pre-incubation	5 minutes at 20°C	5 minutes at 20°C	10 minutes at 4°C
Incubation	120 minutes at 20°C	40 minutes at 20°C	80 minutes at 4°C
Wash	4 minutes at 4°C	4 minutes at 4°C	20 minutes at 4°C
Scatchard plot			
K_d (nM)	2.748	3.704	3.610
B_{max} (fmol/mg protein)	325.59	225.70	236.76

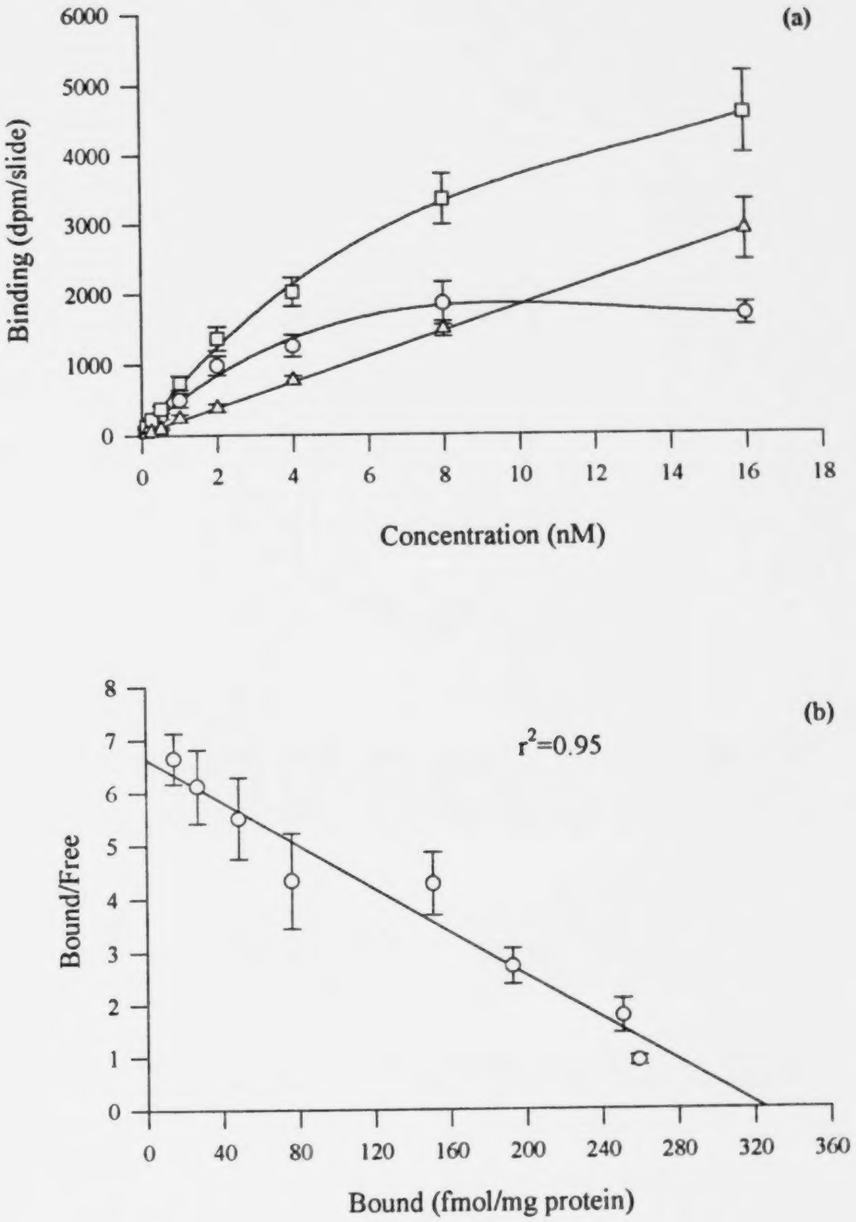
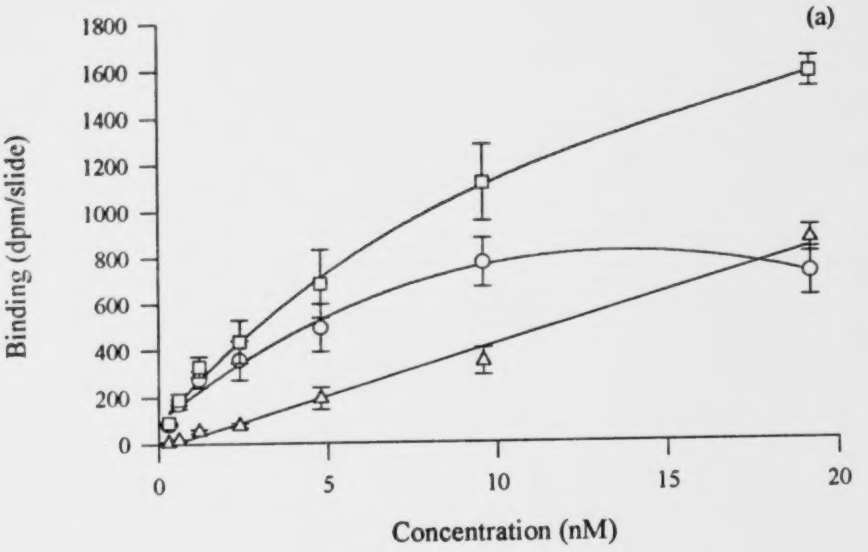


Figure IV.4 Saturation curve (a) and Scatchard plot (b) of [^{3}H]DAGO binding in brain sections of the Columbian ground squirrels. (open square represents total binding, open circle means specific binding, and open triangle indicates non-specific binding, n=3)



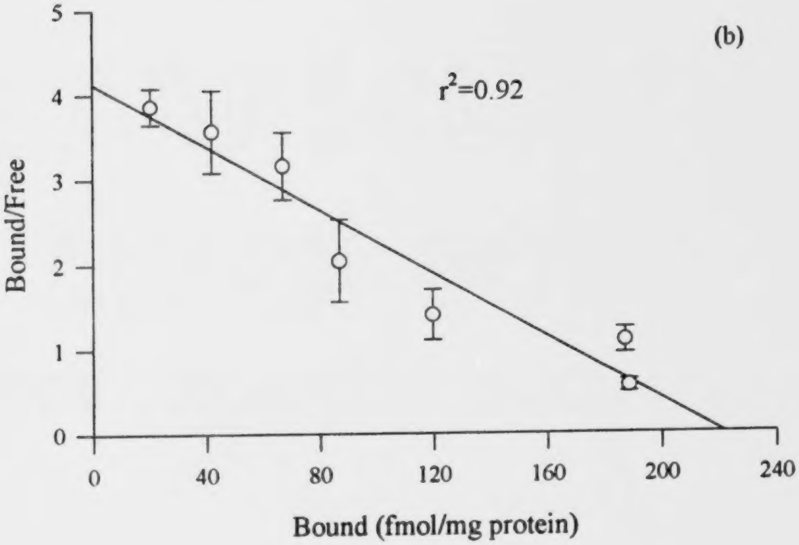


Figure IV.5 Saturation curve (a) and Scatchard plot (b) of [^{3}H]DPDPE binding in brain sections of the Columbian ground squirrels. (open square represents total binding, open circle is specific binding, and open triangle designates non-specific binding, n=3)

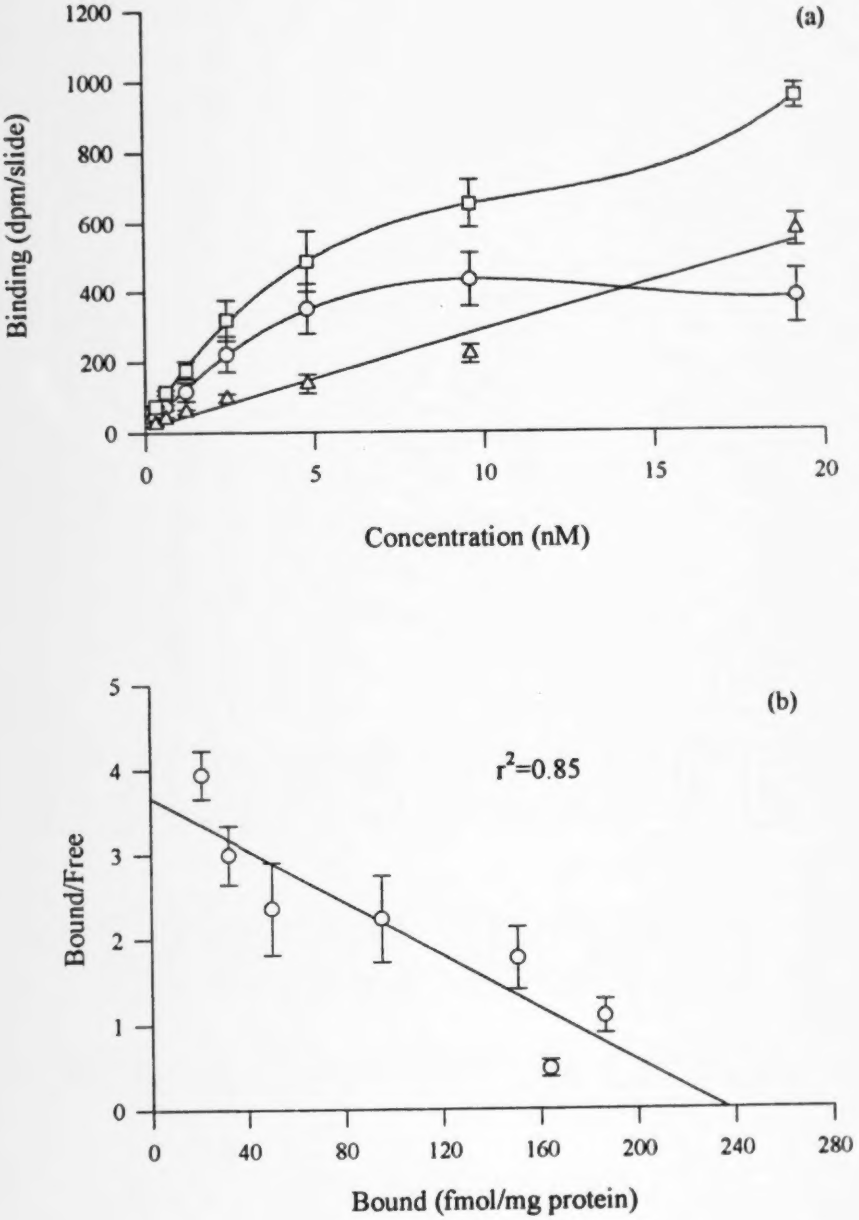


Figure IV.6 Saturation curve (a) and Scatchard plot (b) of [^{3}H]EKC binding in brain sections of the Columbian ground squirrels. (open square indicates total binding, open circle represents specific binding, and open triangle designates non-specific binding, n=3)

Displacement of specific opioid receptor binding by different opioid or non-opioid ligands

To evaluate the receptor specificity of various ligands, brain sections were incubated in 2 nM [^{3}H]DAGO, or 4 nM [^{3}H]DPDPE, or 3 nM [^{3}H]EKC in the presence or absence of other opioid or non-opioid ligands with the conditions listed in Table IV.1. The radioactivity of each slide was then measured and the specific binding of the radioactive ligand in each condition was normalized using the control slices incubated only with the corresponding tritiated ligand as 100%. The percentage of specific binding in each condition was plotted against the concentration of the corresponding cold ligand. The specificity of each ligand to a certain receptor type was expressed as its IC_{50} value which was obtained from the displacement curve.

As demonstrated in Figure IV.7, the μ agonist DAGO displaced about 5% of [^{3}H]DAGO at 0.1 nM, 40% at 1 nM, and 72% at 10 nM. Therefore, its IC_{50} was 3.16 nM. Bremazocine, a general opioid agonist with certain preference to κ receptor, also replaced [^{3}H]DAGO very efficiently: around 5% at 0.1 nM, 47% at 1 nM, 78% at 10 nM, and with an IC_{50} of 1.58 nM. Naloxone, a general opioid antagonist with high μ affinity, had an IC_{50} value of 5.37 nM to displace [^{3}H]DAGO. On the other hand, the δ agonist DADLE, the κ antagonist Nor-BNI, the δ antagonist naltindole, and the κ agonist U50488 had much lower affinities for μ receptors, with IC_{50} values of 25.1 nM, 0.13 μM, 0.18 μM, 0.55 μM to displace [^{3}H]DAGO. In addition, the non-opioid ligand 5-HT could hardly displace any [^{3}H]DAGO binding from the μ receptor even at a concentration of 1 mM.

To displace 50% of the δ receptor from [^{3}H]DPDPE binding, 1.26 nM of DADLE or 2.51 nM of naltrindole was required (Figure IV.8). Other opioid ligands, such as bremazocine, naloxone, Nor-BNI, DAGO, and U50488, were less compatible in displacing [^{3}H]DPDPE (with IC_{50} values of 0.14 μM, 0.18 μM, 0.56 μM, 5.01

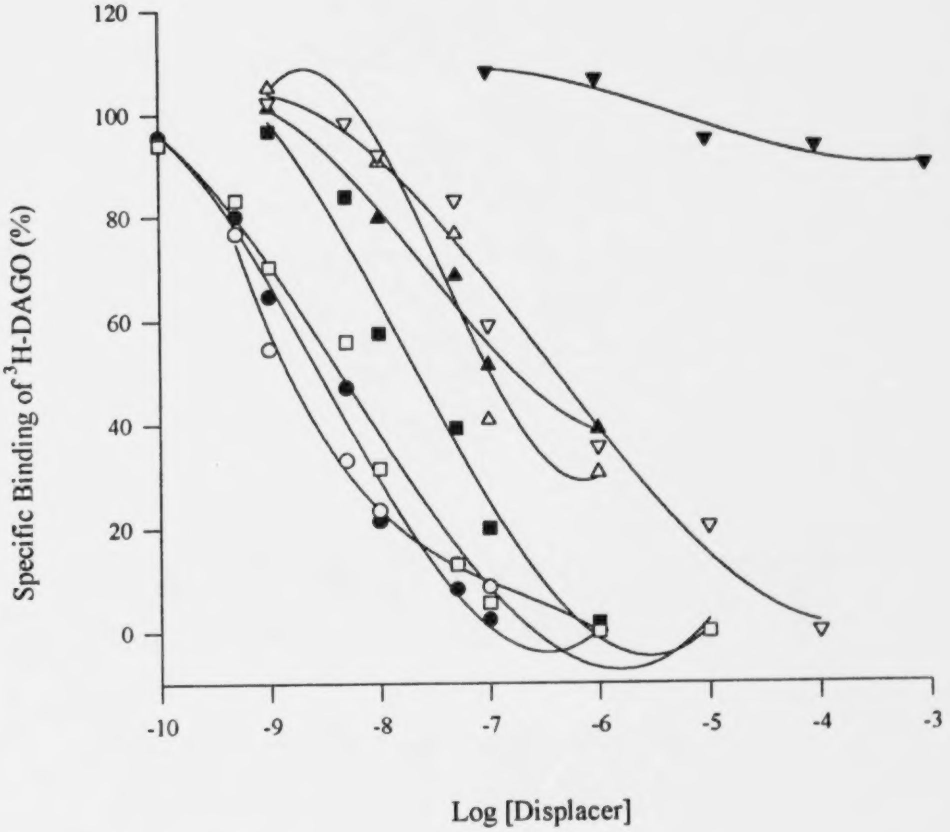


Figure IV.7 Displacement of [^{3}H]DAGO bindnig to μ receptors by various opioid or non-opioid ligands.
(n=3, Legend: open circle, bremazocine, closed circle, DAGO, open square, naloxone, closed square, DADLE, open triangle, Nor-BNI, closed triangle, naltrindole, reversed open triangle, U50488, and reversed closed triangle, 5-HT)

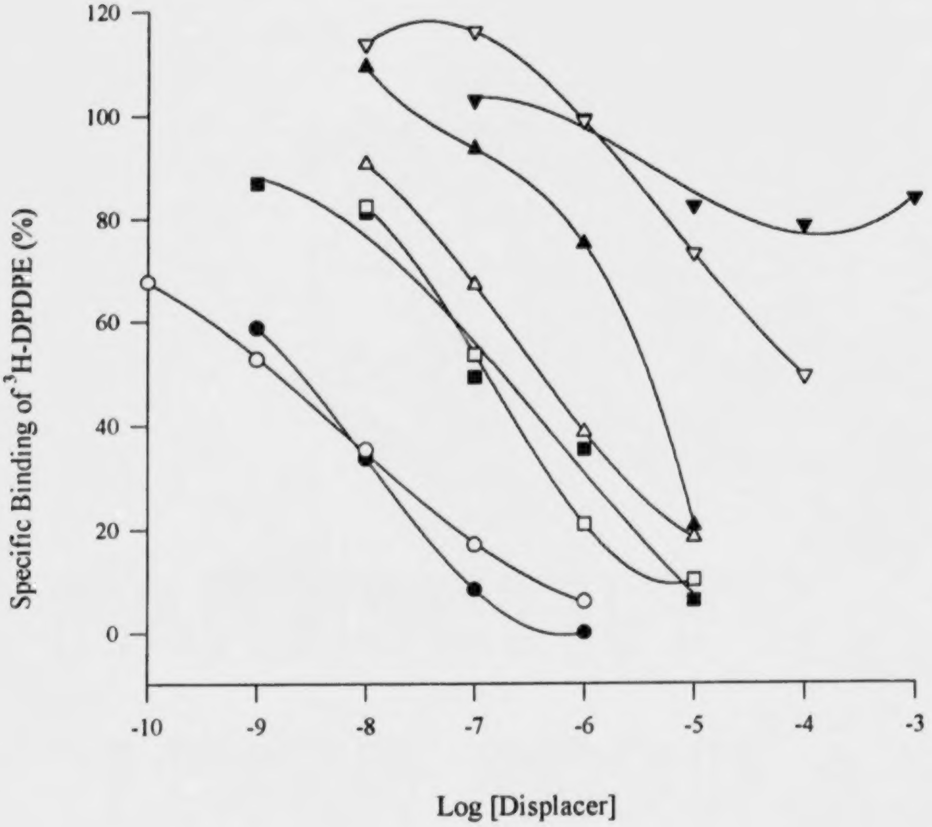


Figure IV.8 Displacement of binding of the δ agonist [^{3}H]DPDPE by different opioid agonists, opioid antagonists, or a non-opioid ligand.
(n=3, Legend: open circle, DADLE, closed circle, naltrindole, open square, bremazocine, closed square, naloxone, open triangle, Nor-BNI, closed triangle, DAGO, reversed open triangle, U50488, and reversed closed triangle, 5-HT)

μM, 89.1 μM, respectively). The non-opioid ligand 5-HT only displaced less than 15% of the [^{3}H]DPDPE binding even at a concentration up to 1 mM.

For [^{3}H]EKC binding to the κ receptor, only 1.05 nM of bremazocine, or 2.82 nM of Nor-BNI, or 8.91 nM of U50488 could displace it by 50% (Figure IV.9). Other opioid ligands, such as DAGO, DADLE, naloxone, and naltrindole, used in the experiments could only displace the [^{3}H]EKC binding by 20% to 40% at a concentration up to 10 μM. Also, the non-opioid ligand 5-HT did not displace any [^{3}H]EKC binding even at a concentration of 1 mM.

Analyses of the Autoradiogram

The μ receptor binding in the limbic system of non-hibernating and hibernating ground squirrels is shown in Figure IV.10. The darkness of a certain region indicated the binding density of [^{3}H]DAGO. As can be seen, μ receptor binding in the limbic region of non-hibernating ground squirrel was densely located in the lateral septum, preoptic area, CA3 region of the hippocampus, and hippocampal fissure, while it was only moderately distributed in the medial septum, CA1 and dentate gyrus of the hippocampus. As for the non-specific binding, the optical density was releative evenly distributed and slightly darker than the background. In calculating the specific binding, the amount of radioactivity of non-specific binding was subtracted from the total binding. During hibernation, the μ receptor binding in the medial septal and CA3 region of the hippocampus was significantly reduced when compared with that of the non-hibernating squirrels (Table IV.2).In the other regions of the limbic system examined, such as the lateral septum, the preoptic area, and dentate gyrus of the hippocampus, there were no significant changes in [^{3}H]DAGO binding between the hibernating and non-hibernating ground squirrels (Table IV.2). The non-specific binding in the brain sections of hibenrating animals was similar to those of non-hibernating animals.

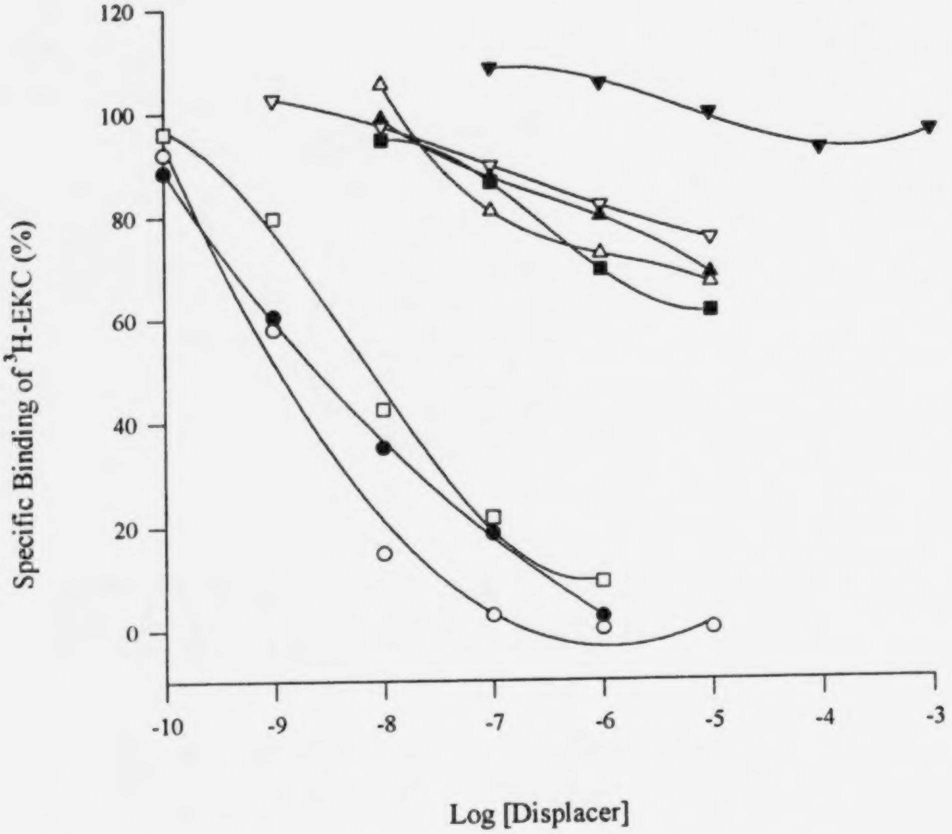
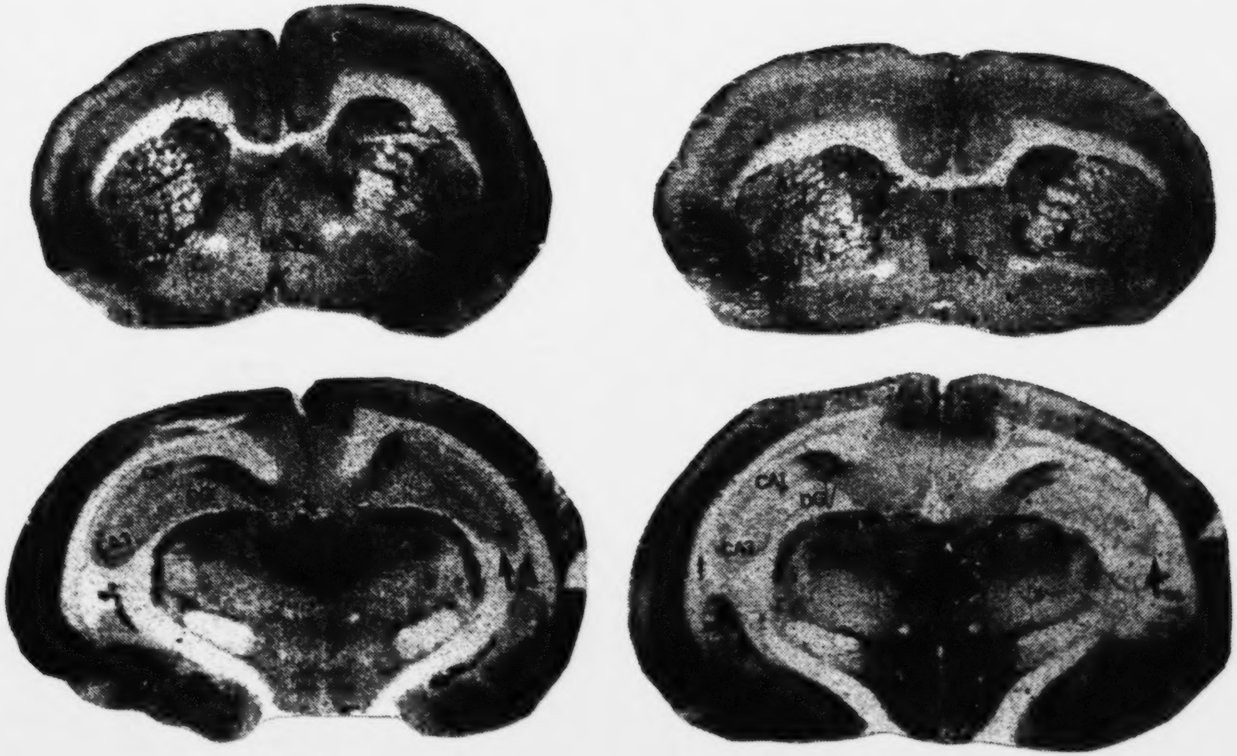


Figure IV.9 Displacement of binding of the [^{3}H]EKC to κ receptors by opioid agonists, opioid antagonists, or a non-opioid ligand.
(n=3, Legend: open circle, bremazocine, closed circle, Nor-BNI, open square, U50488, closed square, DADLE, open triangle, DAGO, closed triangle, naltrindole, reversed open triangle, naloxone, and reversed closed triangle, 5-HT)



Non-hibernating squirrel Hibernating squirrel

Figure IV.10 Autoradiograms of [^{3}H]DAGO binding in certain CNS areas of non-hibernating and hibernating Columbian ground squirrels. Arrows indicate the regions where significant difference between the non-hibernating and hibernating ground squirrels was observed. (MS: medial septum, LS: lateral septum, HF: hippocampal fissure, DG: dentate gyrus, CA1 and CA3: regions in the hippocampal formation)

TABLE IV.2 Density of μ agonist [^{3}H]DAGO specific binding (fmol/mm^2) in the CNS regions of non-hibernating and hibernating Columbian ground squirrels

	Non-hibernating	Hibernating	Mann-Whitney tests P value
Lateral Septum	0.057 ± 0.005	0.054 ± 0.004	0.60
Medial Septum	0.027 ± 0.002	0.010 ± 0.004	0.02*
Preoptic Area	0.049 ± 0.005	0.053 ± 0.005	0.75
CA1	0.030 ± 0.004	0.027 ± 0.004	0.12
CA3	0.050 ± 0.005	0.038 ± 0.005	0.01*
Dentate Gyrus	0.030 ± 0.005	0.029 ± 0.004	0.92
Hippocampal Fissure	0.088 ± 0.013	0.064 ± 0.008	0.35

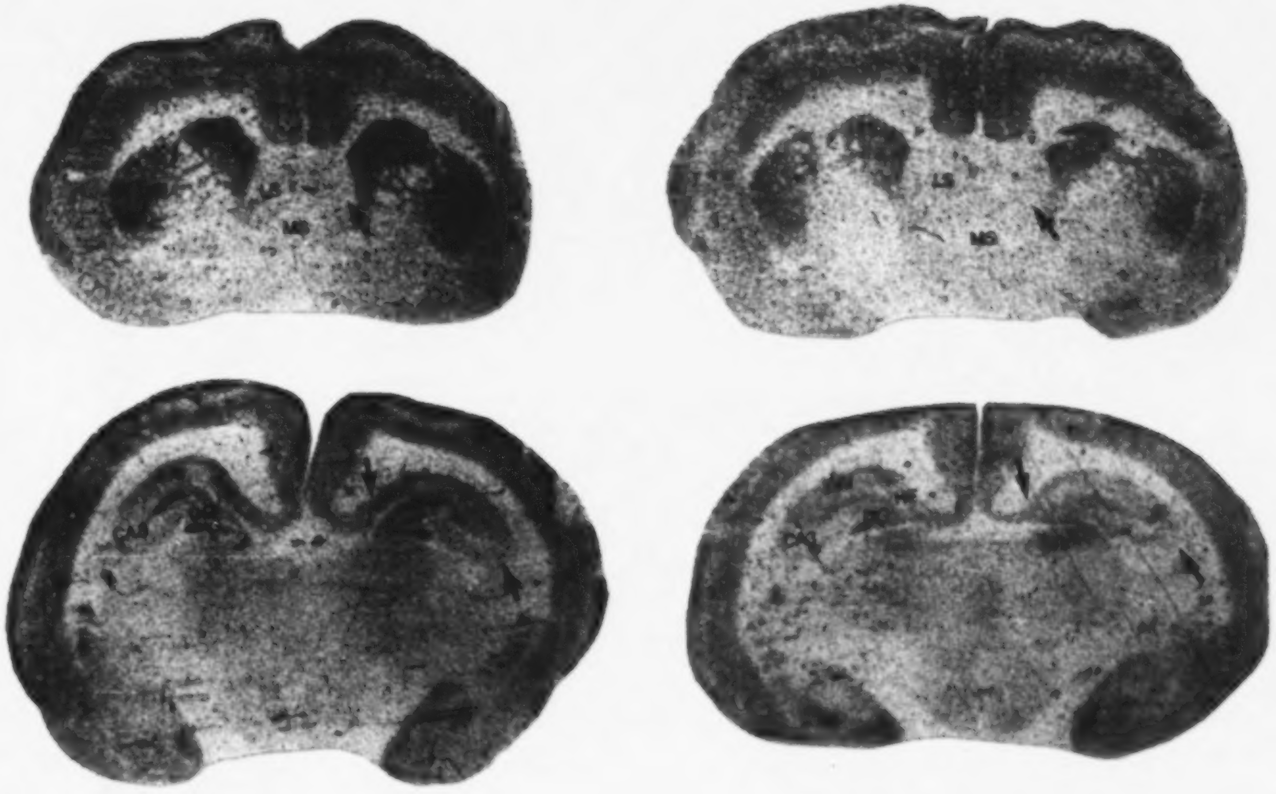
The value represents mean ± s.e. of five animals with quintuplicate measurements in each region of every animal after correcting for non-specific binding. * means the difference between non-hibernating and hibernating ground squirrels is significant at $P < 0.05$.

Comparatively, the overall δ receptor binding in the non-hibernating ground squirrels was less than that of μ binding. Its distribution in the limbic system was mainly concentrated in CA1, dentate gyrus, and hippocampal fissure of the hippocampal formation (Figure IV.11). A moderate localization of δ receptor was also observed in CA3 of the hippocampus, whereas δ receptors were sparsely located in the lateral septum, medial septum, and the preoptic area. The binding of [^{3}H]DPDPE to δ receptor in the lateral septum, CA3, and hippocampal fissure of the hippocampal formation was significantly decreased in the hibernating squirrel. The reduction in δ receptor binding of hibernating ground squirrels was also observed in the CA1 and dentate gyrus of the hippocampus although the difference was not significant ($P=0.074$ and 0.069, respectively, Table IV.3).

Different from μ and δ receptors, the distribution of the κ receptor was limited in certain regions. For instance, it was very dense in the claustrum of the limbic system, while only moderate in dentate gyrus of the hippocampus and sparsely in most other parts of the limbic system, such as the lateral septum, medial septum, the preoptic area, and the CA1 and CA3 of the hippocampus (Figure IV.12). In the hibernating squirrels, the [^{3}H]EKC binding was significantly reduced in the claustrum and the CA3 of the hippocampus (Table IV.4). In the lateral septum and preoptic area, a decrease in κ receptor binding was also observed though not significantly ($P=0.054$ and 0.064, respectively, Table IV.4).

Discussion

Before autoradiography can be performed, the optimal incubation conditions have to be determined for a specific species. Although the washout and pre-incubation procedures were both aimed at dissociating the specific bound ligand from its receptor, the criteria used for evaluating the time required for each were different. Washout was aimed at reducing the non-specific binding of the ligand



Non-hibernating squirrel

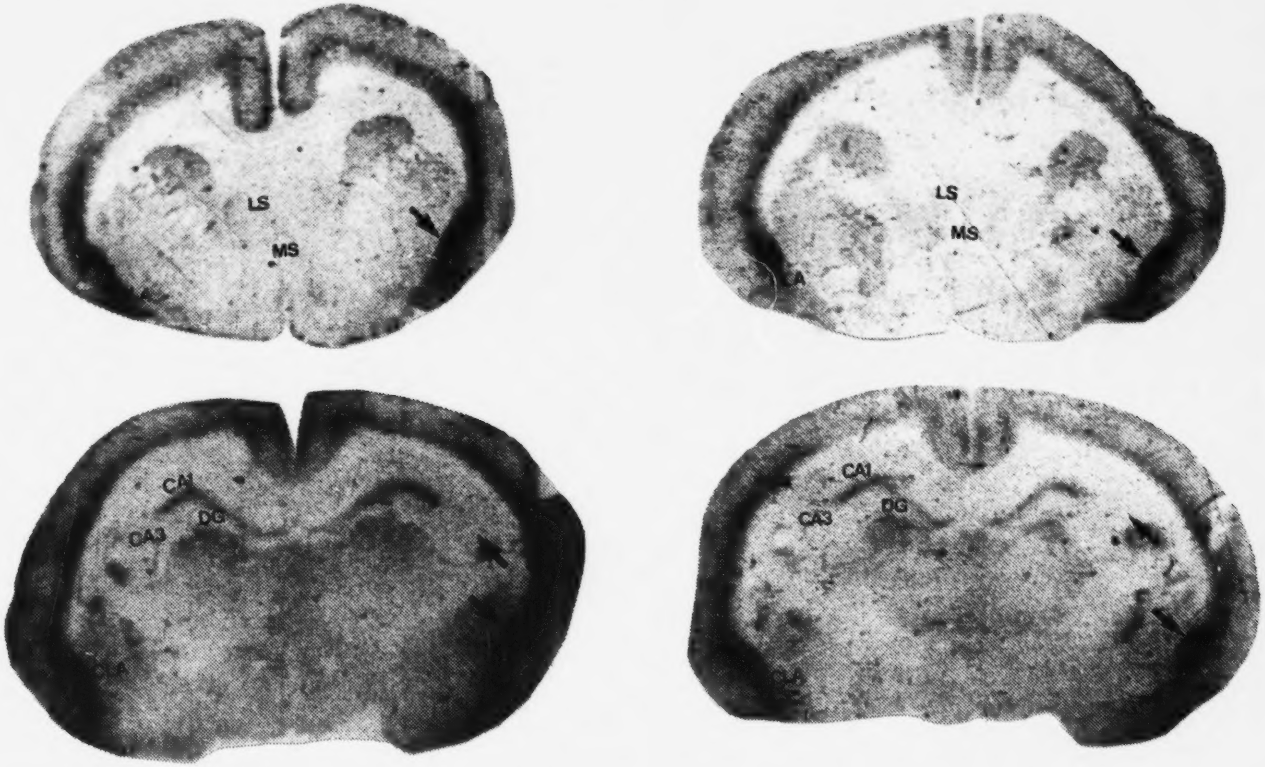
Hibernating squirrel

Figure IV.11 Autoradiograms of [^{3}H]DPDPE binding in certain CNS regions of non-hibernating and hibernating Columbian ground squirrels. Arrows indicate the areas where significant difference between the non-hibernating and hibernating ground squirrels was observed. (MS: medial septum, LS: lateral septum, HF: hippocampal fissure, DG: dentate gyrus, CA1 and CA3: regions in the hippocampal formation)

TABLE IV.3 Density of δ agonist [^{3}H]DPDPE specific binding (fmol/mm^2) in the CNS regions of non-hibernating and hibernating Columbian ground squirrels

	Non-hibernating	Hibernating	Mann-Whitney tests P value
Lateral Septum	0.020 ± 0.004	0.012 ± 0.002	0.03*
Medial Septum	0.010 ± 0.006	0.002 ± 0.003	0.21
Preoptic Area	0.018 ± 0.005	0.014 ± 0.005	0.43
CA1	0.043 ± 0.011	0.032 ± 0.007	0.07
CA3	0.032 ± 0.013	0.023 ± 0.009	0.04*
Dentate Gyrus	0.044 ± 0.007	0.031 ± 0.004	0.07
Hippocampal Fissure	0.051 ± 0.012	0.035 ± 0.005	0.02*

The value represents mean ± s.e. of five animals with quintuplicate measurements in each region of every animal after correcting for non-specific binding. * represents the difference between non-hibernating and hibernating ground squirrels is significant at $P < 0.05$.



Non-hibernating squirrel

Hibernating squirrel

Figure IV.12 Autoradiograms of [^{3}H]EKC binding in several CNS regions of non-hibernating and hibernating Columbian ground squirrels. Arrows indicate the areas where significant difference between the non-hibernating and hibernating ground squirrels was observed. (MS: medial septum, LS: lateral septum, CLA: claustrum, DG: dentate gyrus, CA1 and CA3: regions in the hippocampal formation)

TABLE IV.4 Density of κ agonist [^{3}H]EKC specific binding (fmol/mm^2) in the CNS regions of non-hibernating and hibernating Columbian ground squirrels

	Non-hibernating	Hibernating	Mann-Whitney tests P value
Lateral Septum	0.023 ± 0.008	0.017 ± 0.006	0.05
Medial Septum	0.017 ± 0.004	0.014 ± 0.003	0.14
Preoptic Area	0.021 ± 0.009	0.013 ± 0.006	0.06
CA1	0.021 ± 0.008	0.014 ± 0.004	0.18
CA3	0.023 ± 0.003	0.013 ± 0.001	0.03*
Dentate Gyrus	0.036 ± 0.014	0.026 ± 0.013	0.25
Claustrum	0.199 ± 0.029	0.162 ± 0.021	0.05*

The value represents mean ± s.e. of five animals with quintuplicate measurements in each region of every animal after correcting for non-specific. * means the difference between non-hibernating and hibernating ground squirrels is significant at $P<0.05$.

to a reasonable level without losing too much of the specific binding. The purpose of pre-incubation, on the other hand, was to reduce the pre-occupation of receptors by the endogenous ligand. Therefore, the washout and pre-incubation time may not be the same, they may also be different from the association time as they did not reach the equilibrium (Table IV.1).

Another concern in the autoradiography is whether the specific ligand used in the experiment really binds to its relevant receptor. Therefore, the pharmacological characteristics of each ligand need to be examined. The K_d value of [^{3}H]DAGO binding in the brain of ground squirrels obtained from the Scatchard plot was 2.75 nM which was close to the reported values obtained from other species ranging from 0.20 to 4.5 nM (Wood 1986, Paterson 1991, Fan et al. 1992). The K_d values of [^{3}H]DPDPE and [^{3}H]EKC were 3.70 nM and 3.61 nM respectively, which were also in the reasonable range of the reported values from 0.63 to 4.9 nM and 1.7 to 5.8 nM, respectively (Wood 1986, Paterson 1991, Stevens et al. 1991, Fan et al. 1992). In addition, all three types of receptor binding in the brain of the ground squirrels were determined as having only one high affinity binding-site (using the Beckman "AccuFit Saturation-two site" program for receptor binding). Further displacement experiments indicated that each of the tritiated ligands used indeed bound to its own specific receptor with fairly high selectivity. For instance, [^{3}H]DAGO was only displaced by those ligands with high μ affinity at the nM range, whereas other δ or κ ligands could only displace it at much higher concentration (Figure IV.7) and the μ/δ ratio of DAGO was about 1000 (Figure IV.7 and 8). Similarly, [^{3}H]DPDPE or [^{3}H]EKC binding could only be displaced by low concentrations (nM range) of its specific agonists or antagonists (Figure IV.8, 9). Therefore, the receptors investigated in the experiments were very likely μ, δ, and κ receptors as intended.

To quantify the density of receptor binding in certain brain regions the traditional way, i.e., as fmol/mg protein, a series of standards had to be used.

However, because of the difference in density and physical characteristics between brain and manufactured plastic standards, the quenching factors for tritiated ligand are completely different. Therefore, proper correction of the quenching factor is essential (Kuhar and Unnersrall 1990, Geary et al. 1985) in achieving accurate results. Instead of trying to make quenching corrections of our brain tissue against commercial standards, we used our own standards employing a homogeneous brain paste of the Columbian ground squirrel. To convert the optical density of an autoradiogram to the receptor ligand binding of a specific region as fmol/mg protein by comparing with a standard curve, two assumptions are generally made: (1) the interested brain region is uniform in the density of white matter and grey matter, therefore the quenching factor is identical to that of the standard, and (2) the protein content is also evenly distributed in the region. However, the brain itself is not a homogeneous tissue and the quenching effects of grey matter are different from that of the white matter. Therefore, the quenching factor may vary from one region to another. In addition, the protein content in the brain is not homogenously distributed either. To meet the first assumption, other than using the same tissue to make standards, there is not a good and easy way to get around the problem except removal of lipids by chloroform extraction (Geary et al. 1985). Since the main concern of the present study was to compare changes in receptor binding in the same region under two different physiological conditions, but not the absolute level of receptor binding between regions, the difference in quenching should only be minimal. The second criterion can be met by expressing the data only as fmol per unit area (rather than per mg protein), which has been found to be more reliable (Kuhar and Unnersrall 1990).

Similar to the reported results for the rat brain (Mansour et al. 1988, Sharif and Hughes 1989), the distribution and binding of the three types of opioid receptors in the Columbian ground squirrels' CNS was also quite different. The μ receptor was the densest in binding of the three types and it was evenly distributed throughout the limbic system. The δ receptor binding was less than the μ receptor overall, with most of it concentrated in the hippocampal region. With the exception of a very dense binding in the claustrum, κ receptor binding was the sparsest of the three opioid receptors. During hibernation, however, the density of opioid receptors decreased in certain CNS regions. The reduction in μ receptor binding was not uniform throughout all regions; it was significantly reduced in the medial septum and CA3 region, but without change in the lateral septum, preoptic area and dentate gyrus. Therefore, the decrease in receptor binding in the hibernating ground squirrel was not a uniform depression of the opioid system. This conclusion was supported by a recent study (Beckman et al. 1993) that the failure in development of physical dependence to morphine observed in the hibernating animal can not be mimicked by a general depression of the CNS induced by pentobarbital. Instead, the alteration in receptor binding during hibernation appears to be site- and receptor-specific. The reduction in δ receptor binding was found localized in the lateral septum and the CA3 and hippocampal fissure of hippocampal region, whereas that of κ binding was most profound in the claustrum and CA3 of the hippocampus.

The seasonal changes in opioid receptor sensitivity to exgenous opioids have been shown by previous investigations examining state-dependent changes in thermoregulation (Wang et al. 1987, Lee et al. 1989) and modulation of neurotransmitter release (Kramarova et al. 1991, Cui et al. 1993). Injection of met-enkephalinamide into the lateral septum of non-hibernating ground squirrel induced a biphasic thermoregulatory responses: hyperthermia at low dose and hypothermia at high dose, but only a uniphasic hyperthermia was induced after high dose in the hibernating state. This right-hand shift in the dose-response curve could be explained by the observed reduction in [^{3}H]DPDPE binding to δ receptor in the lateral septum of the hibernating ground squirrels (Table IV.3). In addition, the attenuation of inhibitory effect of δ agonist on 5-HT release from the hippocampal slices of hibernating ground squirrel (Chapter Two) may be a result of the decrease in δ receptor binding in the CA3 and hippocampal fissure of the hippocampal region (about 29%). Similarly, the decline of the density of κ receptor binding by 31% (mainly in CA3 of the hippocampus) may also beaccounted for the attenuation of κ agonist mediated stimulation on 5-HT release from the hippocampal slice of hibernating animals. However, the [^{3}H]DAGO binding in the hippocampal region of hibernating ground squirrel only decreased by 19% (mostly in CA3 region) which lead to the insignificant downward shift of dose-response curve of the inhibitory modulation of μ agonist on 5-HT release (Chapter Two). It has been known for a long time that increased extracellular neurotransmitter will induce desensitization or down-regulation of its receptors. Chronic administration of exogenous opioids have been shown to either desensitize or down-regulate various opioid receptors (Bhargava et al. 1991, Tao et al. 1991). An elevation of endogenous opioid activity could also lead to the downregualtion of opioid receptor (Stein et al. 1992). It is thus quite possible that the observed decrease in opioid receptor binding in specific CNS regions during hibernation may be a result of increased endogenous opioid activity.

Different from the hippocampal region, the μ receptor binding in the medial septum was decreased strikingly (59%) in the hibernating ground squirrels. The medial septum is generally believed to be one of the critical elements in generating the theta-rhythm of the septo-hippocampal complex (Green and Arduini 1954, Petsche et al. 1962, Stewart and Fox 1990). The inhibition of theta-rhythm has been speculated to directly regulate the entry and maintenance of hibernation (Heller 1979). As the nature of opioid activation is mainly inhibitory, an increase in endogenous μ activity in the medial septum may suppress neuronal activity (including theta-rhythm) in the septo-hippocampal complex, facilitating the onset or maintenance of hibernation. On the other hand, down-regulated opioid receptors, in the late phase of a hibernation bout, in concert with other excitatory neurotransmitters, e.g. norepinepherine and thyrotropin-releasing hormone, may lead to reversal of the inhibition in the septo-hippocampal region and induce arousal from hibernation (Beckman and Stanton 1982, Belousov and Belousova 1992, Belousov 1993). The increase in inhibitory μ activity in hibernation, therefore, further supports the theory that the medial septum is an important functional link in regulating different states of the hibernation cycle (Belousov 1993).

Another interesting finding was the significant reduction in the binding of κ receptor in the claustrum of hibernating animals. Although the exact function of this nucleus is still largely unknown, it has been suspected to be responsible for appetite regulation (Simpson and Fitch 1988). κ agonists have been demonstrated to be involved in feeding and body weight gain (Ferguson-Segall et al. 1982, Morley and Levine 1981, 1983). In addition, a seasonal increase in the activity of the endogenous κ agonist, dynorphin, has been observed in ground squirrels (Nizielski et al. 1986). It is thus quite possible that an increase in κ activity, indicated by down-regulation of κ receptors, in the claustrum may be related to the pre-hibernation fattening.

To date, the exact mechanisms which regulate or cause the changes in receptor binding are unclear. It can be due to an increase in endogenous opioid activity by an endogenous clock, which in turn down-regulates their receptors. However, the expression of opioid receptors has also been demonstrated to be modulated by other neurotransmitters and second messenger systems (Desjardins et al. 1992, Shariabi and Sharp 1993, Simth et al. 1993). The possibility of a spontaneous reduction in the expression of opioid receptor controlled by an endogenous mechanism cannot be excluded until further investigation of opioid receptor gene expression by *in situ* hybridization has been carried out. Nevertheless, the results tend to suggest that the non-uniform changes in binding to various opioid receptors may be responsible for their diverse physiological roles in regulating different states of hibernation.

References

Akil H., Bronstein D., and Mansour A. (1988) Overview of the endogenous opioid systems: anatomical, biochemical and functional issues. Endorphins, Opiates and Behavioral Processes Rodgers R.J. and Cooper S.J. (eds), pp 1-23, Chichester, New York.

Aloia R.C., Vasquez B.J., and Tucker S.L. (1985) Opioid receptors in the brain of active and hibernating ground squirrels, *Spermophilus lateralis.* Abstracts for Living in the Cold. (Fallen Leaf Lake, California) Heller H.G., Musacchia X.J., and Wang L.C.H. (eds), p 211

Atweh S.F. and Kuhar M.J. (1983) Distribution and physiological significance of opioid receptors in the brain. Br Med Bull 39:47-52

Barg J. and Simantov R. (1991) Transient expression of opioid receptors in defined regions of developing brain: are embryonic receptors selective? J Neurochem 57:1978-84

Beckman A.L. and Stanton T.L. (1982) Properties of the CNS during the state of hibernation. The Neural Basis of Behavior Beckman A.L. (ed), pp 19-45, Spectrum, New York.

Beckman A.L., Dean R.B., and Wamsley J.K. (1986) Hippocampal and cortical opioid receptor binding: changes related to the hibenrnation state. Brain Res 386:223-231

Beckman A.L., Beaver T.A., and Lewis F.A. (1993) Morphine physical dependence in the hibernator: cental nervous system mechanisms underlying the development of dependence remain functional during depression induced by pentobarbital anaesthesia. Life Sci 52:1079-1086

Belousov A.B. and Belousova J.V. (1992) State-dependent and state-independent excitibility of the medial septal neurons in brain slices of ground squirrel, *Citellus undulatus.* Neuroscience 38:599-608

Belousov A.B. (1993) On possible mechanisms of the septohippocampal system participation in the neural control of hibernation. Life in the cold: Ecological, physiological, and molecular mechanisms. Carey C., Florant G.L., Wunder B.L., and Horwitz B. (eds), pp 291-296, Westview Press, Colorado.

Bhargava H.N., Gulati A., and Ramarao P. (1991) Down-regulation of brain and spinal cord κ-opiate receptors in spontaneously hypertensive, Wistar-

Kyoto normotensive, and Sprague-Dawley rats by chronic treatment with U50488H. Biochem Pharmacol 42:25-29

Bradford M.M. (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 27:248-254

Chang K.J. (1984) Opioid receptors: mutiplicity and sequelae of ligand-receptor interactions. The Receptors Vol.1, Conn M. (ed), pp 1-81, Academic Press, New York.

Cui Y., Lee T.F., Kramarova L.I., and Wang L.C.H. (1993) The modulatory effects of *mu* and *kappa* opioid agonists on 5-HT release from hippocampal and hypothalamic slices of euthermic and hibernating ground squirrels. Life Sci 53:1957-1965

Desjardins G.C., Brawer I.R., and Beaudet A. (1992) Monosodium glutamate-induced reductions in hypothalamic beta-endorphin content result in mu-opioid receptor upregulation in the medial preoptic area. Neuroendocrinology 56:378-384

Dingledine R., Valentino R.J., Bostock E., King M.E., and Chang K.J. (1983) Downregulation of δ but not μ opioid receptors in the hippocampal slice associated with loss of physiological response. Life Sci 33 Suppl 1:333-336

Duka T., Schubert P, Wuster M, Stoiber R., and Herz A. (1981) A selelctive distribution pattern of different opioid receptors in certain areas of rat brain as revealed by in vitro autoradiography. Neurosci Lett 21:119-124

Fan S.F., Shen K.F., Scheideler M.A., and Crain S.M. (1992) F11 neuroblastoma X DRG neuron hybrid cells express inhibitory mu- and delta-opioid receptors which increase voltage-dependdent K^+ currents upon activation. Brain Res 590:329-333

Ferguson-Segall M., Flynn J.J., Walker J., and Margules D.L. (1982) Increased immunoreactive dynorphin and leu-enkephalin in posterior pituitory of obese mice (*ob/ob*) and super-sensitivity to drugs that act at *kappa* receptors. Life Sci 31:2233-2236

Geary E.A.III, Toga A.W., and Wooten G.F. (1985) Quantitative film autoradiography for tritium: methodological considerations. Brain Res 337:99-108

Gilbert P.E., and Martin W.R. (1976) The effects of morphine- and nalorphine-like drgus in the nondependent, morphine-dependent and cyclazocine-dependent chronic spinal dog. J Pharmacol Exp Ther 198:66-82

Green J.D. and Arduini A.A. (1954) Hippocampal electrical activity in arousal. J Neurophysiol 17:533-557

Heller H. C. (1979) Hibernation: neural aspects. Ann Rev Physiol 41:305-321

Herkenham M. and McLean S. (1988) The anatomical Relationship of opioid peptides and opioid receptors in the hippocampi of four rodent species. Opioids in the Hippocampus McGinty J.F. and Friedman D.P. (eds), pp 33-47, NIDA publication, Rockville.

Khachaturian H., Lewis M.E., Schafer M.K.H., and Waston S.J. (1985) Anatomy of the CNS opioid system. TINS 8:111-119

Kolaeva S.G., Kramarova L.I., and Yarying K.N. (1989) The role of opioids and TRH in the hibernation bout/arousal cycle of ground squirrels *Citellus undulatus*. Living in the cold II Malan A. and Canguilhem B. (eds), pp 461-466, John Libbey Eurotext, Montrouge.

Kramarova L.I., Lee T.F., Cui Y., and Wang L.C.H. (1991) State-dependent variation in the inhibitory effect of [D-Ala^2,,D-Leu^5]-enkephalin on hippocampal serotonin release in ground squirrels. Life Sci 48:175-181

Kukar M.J. and Unnerstall J.R. (1990) Receptor autoradiography. Methods in Neurotransmitter Receptor Analysis Yamamura H.I., Enna S.J., and Kuhar M.J. (eds), pp 179-218, Raven Press, New York.

Lee T. F., Nurnberger F., Jourdan M. L. and Wang L. C. H. (1989) Possible involvement of septum in seasonal changes in thermoregulatory responses to met-enkephalinamide in ground squirrels. Thermoregulation: Research and clinical applications Schonbaum E. and Lomax P. (eds), pp 200-203, Kruger, Basel.

Loh H.H and Smith A.P. (1990) Molecular characterization of opioid reecptors. Annu Rev Pharmacol Toxicol 30:123-147

Lord J.A.H., Waterfield A.A, Hughes J., and Kosterlitz H.W. (1977) Endogenous opioid peptides: mutiple agonists and rceptors. Nature 267:495-499

Mansour A., Khachaturian H., Lewis M.E., Akil H., and Watson S.J. (1988) Anatomy of CNS opioid receptors. TINS 11:308-314

Martin W.R., Eades C.G., Thompson J.A., Huppler R.E., and Gilbert P.E. (1976) The effects of morphine- and nalorphine-like gruds in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther 197:518-532

McConnaughey M.M., Wong S.C., and Ingenito A.J. (1992) Dynorphin receptor changes in hippocampus of the spontaneously hypertensive rat. Pharmacology 45:52-57

Morley J.E. and Levine A.S. (1981) Dynorphin-(1-13) induces spontanous feeding in rats. Life Sci 29:1901-1903

Morley J.E. and Levine A.S. (1983) Involvement of dynorphin and *kappa* opioid receptors in feeding. Peptides 4:797-800

Nizielski S.E., Levine A.S., Morley J.E., Hall K.A., and Gosnell B.A. (1986) Seasonal variation in opioid modulation of feeding in the 13-lined ground squirrel. Physiol Behav 37:5-9

Paterson S.J. (1991) Opioid receptors. LTP, Galanin, Opioids, Autonomic, 5-HT Stone T.W. (ed), pp 117-140, Taylor & Francis, London.

Pert C.B. and Snyder S.H. (1973) Opiate receptor: demonstartion in nervous tissue. Science 179:1011-1014

Petsche H., Stumpf C.H., and Gogolak G. (1962) The significance of the rabbit"s septum as a relay station between the midbrain and the hippocampus I. the control of hippocampus arousal activity by the septum cells. EEG Clin Neurophysiol 14:202-211

Robson L.E., Gillan M.C.G., and Kosterlitz H.W. (1985) Species differences in the concentration and distribution of opioid binding sites. Eur J Pharmacol 112:65-71

Sharif N.A. and Hughes J. (1989) Discrete mapping of brain *mu* and *delta* opioid receptors using selective peptides: quantitative autoradiography, species differences and comparison with *kappa* receptors. Peptides 10:499-522

Sharabi N.A. and Sharp B.M. (1993) Activation of protein kinase C rapidly down-regulates naloxone-resistant receptors for beta-endophin on U937 cells. J Pharmacol Exp Ther 264:276-281

Simon E.G., Hiller J.M. and Edelman I. (1973) Stereospecific binding of the potent narcotic analgasic [^{3}H]etorphine to rat-brain homogenate. Proc

Natl Acad Sci 70:1947-1949

Simon E.J. (1991) Opioid receptors and endogenous opioid peptides. Med Res Rev 11:357-374

Simpson J.A. and Fitch W. (1988) Applied Neurophysiology Wright, London.

Smith .A., Leslie F.M., Broide R.S., and Loughlin S.E. (1993) Long-term changes in striatal opioid systems after 6-hydroxydopamine lesion of rat substantia nigra. Neuroscience 55:935-951

Stein E.A., Hiller J.M, and Simon E.J. (1992) Effects of stress on opioid receptor binding in the rat central nervous system. Neuroscience 51:683-690

Stevens C.W., Lacey C.B., Miller K.E., Elde R.P, and Seybold V.S. (1991) Biochemical characterization and regional quantification of mu, delta, and kappa opioid binding sites in the rat spinal cord. Brain Res 550:77-85

Stewart M. and Fox S.E. (1990) Do septal neurons pace the hippocampal theta rhythm? TINS 13:163-168

Tao P.L., Tsai C.L, Chang L.R., and Loh H.H. (1991) Chronic effect of [D-Pen^2,D-Pen^5]enkephalin on rat brain opioid receptors. Eur J Pharmacol 201:2009-214

Terenius L. (1973) Characteristics of the "receptor" for narcotic analgesia in synaptic plasma membrane fraction from rat brain. Acta Pharmacol Toxicol 33:377-384

Ueda H., Nozaki M., and Satoh M. (1991) Mutiple opioid receptors and GTP-binding proteins. Comp Biochem Physiol 98C:157-169

Wang L. C. H., Lee T. F., and Jourdan M. L. (1987) Seasonal difference in thermoregulatory responses to opiates in a mammalian hibernator. Pharmacol Biochem Behav 26:565-571

Wang L.C.H. (1993) Is endogenous opioid involved in hibernation? Life in the cold: Ecological, physiological, and molecular mechanism. Carey C., Florant G.L., Wunder B.L., and Horwitz B. (eds), pp 297-304, Westview Press, Colorado.

Wood P.L. (1986) Multiple opioid receptors in the central nervous system. Neuromethods 4: Receptor binding Boulton A.A. , Baker G.B., and Hadina P.D. (eds), pp 329-364

Zukin R.S. and Zukin S.R. (1981) Multiple opiate receptors: emerging concepts. Life Sci 29:2681-2690

CHAPTER FIVE

State-dependent changes of endogenous opioids in the central and peripheral systems in the hibernation cycle

Introduction

Since the discovery of endogenous opioids in the early 1970's (Hughes et al 1975), three distinct endogenous opioid families have been identified, namely: proopiomelanocortin (POMC), proenkephalin, and prodynorphin (Cuello 1983, Khachaturian et al. 1985, Civelli et al. 1986). The post-translational products of POMC gene are β-endorphin, a potent agonist at both μ and δ receptors, and non-opioid peptides ACTH and γ-LPH. Met-enkephalin and leu-enkephalin are the products of the proenkephalin gene expression. Both of them prefer δ receptors. Dynorphin A 1-17, 1-13, 1-8, along with dynorphin B and neo-endorphin, are produced from the prodynorphin family. Most of the dynorphins have κ preference except dynorphin A 1-8 which retains δ capability and dynorphin A 1-13 which has high potency to both κ and μ receptors (Akil et al. 1984, 1988, Brush and Shain 1989, Simon 1991). The diverse endogenous opioids are distributed widely in the central and peripheral nervous systems and several organs or organ systems, such as the adrenal gland, gastrointestinal tract, pancreas, etc. (Hokfelt et al. 1984, Genazzani et al. 1984, 1989,

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Schick and Schusdziarra 1985, Negri et al. 1989, Stengaard-Pedersen 1989). Each type of opioid participates in controlling different physiological functions by activating its specific opioid receptors (Olson et al. 1990, 1992).

Opioids have been indicated to mediate increased feeding at low doses (Gosnell 1987, Stanley et al. 1989) and anorexia at high doses (Yim and Lowy 1984, Nizielski et al. 1986), bradycardia and hypotension (Kunos et al. 1988, Rabkin 1989), respiratory suppression (Yeadon and Kitchen 1989), lowering of set-point (Tset) in thermoregulation (Spencer et al. 1990 Burks 1991, Handler et al. 1992), and releasing of other neurotransmitters (Jackisch et al. 1988, Illes 1989). These opioid-mediated physiological changes are similar to those observed during mammalian hibernation, e.g. hyperphagia induced obesity followed by anorexia, decrease in heart and respiratory rate, and decrease in Tset for body temperature (Tb) regulation. It is, thus, speculated that endogenous opioids may be involved in controlling the hibernation cycle. Previous reports have demonstrated an increase in met-enkephalin-like peptides in the brain extracts (Kromarova et al. 1983) and in the perikarya of the septal and hypothalamic regions (Nurnberger et al. 1991) in hibernation and a decrease in opioid receptor efficacy in hibernation (Beckman et al. 1981, 1986, Wang et al. 1987, Lee et al. 1989). These observations imply an increase in endogenous opioid activity associated with hibernation. However, previous investigations only covered a few regions in the central nervous system (CNS). No attempts have been made to reveal changes, if any, in the peripheral opioid system which has been shown to be generated and regulated differently from the central opioids (Alessio et al. 1989, Negri et al. 1989, Hokefelt et al. 1984). The present study, therefore, aimed to provide a detailed and systematic documentation of changes in endogenous opioids from three opioid families in the central and peripheral systems in different stages of a hibernation season. With the aid of *in vivo* microdialysis (Benveniste 1989, Kendrick 1989, Ungerstedt 1991), we attempted to verify whether the observed changes in opioid content in different regions of the CNS are of any functional significance by simultaneously measuring the extracellular concentration and "apparent turnover" (which is the amount of opioids diffused into the dialysate per unit time) of the same opioids in the same region.

Methods

All experimental protocols had been approved by the University of Alberta Animal Use Committee, following the guidelines of the Canadian Council on Animal Care.

Animals Columbian ground squirrels (*Spermophilus columbianus*) of both sexes were live trapped in the foothills of the Rocky Mountains in Alberta. All squirrels were housed at 22°C under natural photoperiod with free access to food (rodent chow, mixed grains, and sunflower seeds) and water. Their body weights were measured weekly and monitored year round. The onset of hibernation season was determined as a rapid gain in body weight, because of hyperphagia, followed by weight plateau and anorexia. When their body weights reached plateau, the animals were transferred to a cold (4 ± 1°C), dark walk-in environmental chamber to facilitate hibernation. Their activity was checked and recorded daily. The hibernating state was verified as a lack of movement, slow and regular breathing (less than one per minute), and the sawdust remaining on the squirrel's back when placed on it the previous day. The hibernating ground squirrels used for collecting brain tissues and plasma samples were those who had completed at least two hibernation bouts. They were sacrificed on the second day of a hibernation bout while still hibernating (Tb=4-5°C). Non-hibernating squirrels were those whose body weight was relatively constant for at least two months prior to use and were sacrificed while euthermic (Tb=37-

38°C). For microdialysis studies, hibernating squirrels were cannulated during their inter-bout euthermia and used on the second day of a reentered hibernation bout following surgery. Non-hibernating squirrels were used only one week after surgery.

Preparation of brain tissues and plasma samples Non-hibernating or hibernating squirrels were terminated between 10:00 and 11:00 am by decapitation. Brains were rapidly removed from the skull and immediately dissected on ice into several brain regions: frontal cortex, septum, striatum, hypothalamus, claustrum, hippocampus, cerebellum, and medulla. Following separation, the brain tissues were immediately frozen in dry ice and stored at -70°C until used. Simultaneously, about 10 ml blood of the animals were collected in an ice-cold centrifuge tube containing 200 ul of 0.5 mM EDTA solution and 100 μl enkephalinase inhibitors (2 mM bestatin, 100 μM captopril, and 10,000 units aprotinin). Plasma was separated by centrifugation at 3000 rpm for 15 minutes at 4°C and stored at -70°C until used.

The brain tissues were homogenized in 1 ml of 0.1% trifluoroacetic acid (TFA) containing enkephalinase inhibitors: 20 μM bestatin, 1 μM captopril, and 1000 units aprotinin, using an ultrasound homogenizer Sonifier (Branson). An extra 200 μl 10% TFA was added to the homogenate following homogenization. 1 ml of plasma sample was also mixed with 200 μl of 10% TFA. The acidified brain tissue homogenates and plasma were centrifuged at 14,000 rpm for 10 minutes. The pellets were redissolved in 0.1 M perchloric acid and used for measuring the amount of protein (Bradford 1976). Endogenous opioids were extracted from the supernatant using solid phase extraction (SPE). Briefly, C_{18} SPE columns (containing 100 mg packing material) were equilibrated by washing with three 1-ml portions of 60% acetonitrile (ACN) in 0.1% TFA followed by six 1-ml volumes of 0.1% TFA under negative pressure. The supernatant of acidified brain tissue homogenates or plasma were then loaded onto the pre-treated SPE columns and slowly drawn through the column. The column was then washed with two 1-ml volumes of 0.1%TFA. Endogenous opioids from brain tissues or plasma samples were finally eluted with two 1-ml portions of 60% ACN in 0.1% TFA and lyophilized using a Centrivap Concentrator (LABCONCO). The lyophilized extractions were rehydrated either in radioimmunoassay (RIA) buffer (0.05 M NaCl, 0.1% BSA, 0.01% NaN_3, and 0.1% Triton X-100 in 0.1 M sodium phosphate buffer, pH=7.4) for detection of dynorphin A and β-endorphin or in HPLC mobile phase (30% ACN in 20 mM KH_2PO_4, pH 2.7) for analyzing met-enkephalin and leu-enkephalin content. Standard met-enkephalin, leu-enkephalin, dynorphin A, and β-endorphin were underwent the same procedure for estimating the recovery rates of each opioid following SPE extraction. These recovery rates were used for calculating actual content of endogenous opioids.

HPLC detection of met-enkephalin and leu-enkephalin content in the brain tissue and plasma Reverse-phase HPLC with C_{18} column (CSC-Spherisorb-ODS2, 5 μm, 15 x 0.46 cm) coupled with an electro-chemical detector (ESA Coulochem model 5100A) was used to quantify the amount of met-enkephalin and leu-enkephalin. The mobile phase contained 30% ACN in 20 mM KH_2PO_4 (pH 2.7) and was delivered at a flow rate of 1.0 ml/min. Before reaching the injector, the mobile phase was "cleaned" by a high-potential guard cell set at 0.85 V. The settings of the dual analytical detectors were 0.40 V for detector 1 and 0.80 V for detector 2. DADLE, used as an external standard, was mixed with standard met-enkephalin and leu-enkephalin. Brain and plasma samples were spiked with a mixture of 1 ng met-enkephalin, 1 ng leu-enkephalin, and 0.5 ng DADLE before injection and the peaks were compared with the same amount of standard and the unspiked samples to calculate the amount of met-enkephalin and leu-enkephalin in the samples.

Cannulation of Columbian ground squirrels in the septal and hippocampal regions Both non-hibernating and hibernating (in inter-bout euthermia)

Columbian ground squirrels were cannulated unilaterally with 19-gauge stainless steel guide cannulae in the lateral septum and hippocampus under halothane anaesthesia. Briefly, the base skull plane of ground squirrels was re-adjust to the horizontal zero plane by tilting the incisor bar downward 35°. Using interaural line as the zero point, the stereotaxic coordinates for the septum were AP=13 X CR mm, L=0.7 X CR mm, H=3 X CR mm and AP=10 X CR-1 mm, L=4 X CR mm, H=3 X CR mm for the hippocampus. The CR (correction factor) was the distance between the interaural line and the incisor bar divided by 36.8 mm (average distance of all animals used in preparing the atlas). The tips of the guide cannulae were positioned 2 mm above the intended perfusion regions and fixed on the skull with dental cement. A 23-gauge stylet was always kept in the guide cannula when the cannula was not in use. The precise location of the cannula was determined histologically after completion of the experiments.

In vivo microdialysis Microdialysis probes were assembled using hollow dialysis fibre (molecular weight cut off 6000 Dalton, O.D. 250 μm, Spectrum), fused silica capillary tubing (World Precision Instruments Inc.), 23-gauge thin-wall stainless steel tubing (Small Parts Inc.), and PE-50 tubing (Clay Adams) as indicated in Chapter Two. Briefly, a piece of fused silica capillary tubing (about 25 mm in length), as the inlet tubing, was inserted into a 18 mm long 23-gauge stainless-steel tubing. One end of the silica tubing extruded the steel tubing by 1.9 mm and enclosed by a piece of one-end-sealed (epoxy glue) hollow dialysis fibre (10 mm in length) leaving about 0.1 mm space between the sealed end of dialysis fibre and the sealed tip of silica tubing. The remaining part of the dialysis fibre was inserted into the stainless steel tubing and affixed to the steel tubing using epoxy glue. The other end of the fused silica tubing pierced through a PE-50 tubing (10 mm), which was connected to the other end of the 23-gauge stainless-steel tubing for about 3 mm. A small piece of plastic blue tip was finally placed around the joint area of the PE-50, stainless-steel tubing, and fused silica tubing and filled with epoxy glue to strengthen the joint.

Recovery rates of opioids from perfusate of the microdialysis probe was assessed at both 37°C and 5°C by immersing the tip of the probe into various concentrations of dynorphin or β-endorphin in an artificial CSF medium (NaCl 128 mM, KCl 2.55 mM, $CaCl_2$ 1.26 mM, $MgCl_2$ 0.94 mM). The flow rate of microdialysis was also varied to evaluate its effects on absolute recovery (the amount of substance picked up in the perfusate per unit time) and relative recovery (percentage of substance picked up in the perfusate comparing to the bathing medium in which the probe was kept).

On the day of experiment, non-hibernating squirrels were placed in the lab for at least two hours before experimentation to allow them to become accustomed to the environment. A microdialysis probe was inserted into either the septum or the hippocampus and perfused with artificial CSF at a flow rate of 2 μl/min using a CMA/100 Microinjection pump (Carnegie Medicine). Samples were collected every 20 minutes and stored at -70°C. Hibernating squirrels were used on the second day of a hibernation bout. They were gently picked up and rotated so that they lay on their sides. Attempts were made to minimize disturbance when picking up the animals and no other manipulations were involved throughout the experimental period. A microdialysis probe was inserted into the guide cannula in the lateral septum when they were still hibernating (brain temperature, Tbr=4-5°C) and perfused with artificial CSF at 1 μl/min in the cold room (ambient temperature, Ta=4±1°C). A fine thermocouple was introduced into the cannula implanted in the hippocampus to monitor the Tbr. The Tbr, breathing pattern, and activity of the animal were monitored throughout the experiment. Samples were collected every 40 minutes and immediately frozen on dry ice and stored at -70°C. Extracellular concentrations were calculated using the pre-determined relative recovery rates at corresponding temperatures and flow rates. Apparent turnover was obtained using opioid concentration (fmol/μl) in the perfusate multiplying by the flow rate used.

RIA measurement of dynorphin and β-endorphin Dynorphin and β-endorphin in the dialysis perfusate and in extracted brain and plasma samples were analysed using the dynorphin A 1-13 (porcine) RIA kit (RIK8676, Peninsula Inc.) and the β-endorphin (rat) RIA kit (RIK8883, Peninsula Inc.), respectively. The cross-reactivity of dynorphin A 1-13 (porcine) antiserum is: dynorphin A 1-13 (porcine) (100%), Big dynorphin 1-24 (porcine) (100%), Big dynorphin 1-32 (porcine) (25%), dynorphin A (porcine) (100%), dynorphin A 1-12 (porcine) (100%), dynorphin A 1-11 (porcine) (1.5%), dynorphin A 1-10 amide (porcine) (1.5%), dynorphin A 1-9 (porcine) (0%), dynorphin A 1-8 (porcine) (0%), dynorphin A 1-7 (porcine) (0%), dynorphin A 1-6 (porcine) (0%), dynorphin B (porcine) (0%), β-endorphin (0%), and met-enkephalin (0%). The cross-reactivity of β-endorphin (rat) antiserum is: β-endorphin (rat) (100%), β-endorphin (human) (40%), β-endorphin (porcine) (100%), β-endorphin (equine) (100%),-β-endorphin (camel, bovine, ovine) (100%), α-endorphin (100%), γ-endorphin (60%), ACTH (human) (0%), met-enkephalin (0%), γ-MSH (0%). A 20 μl dialysis perfusate spiked with 0.5 pg standard dynorphin A 1-13 or 1 pg β-endorphin was used to measure the dynorphin or β-endorphin content in the perfusate.

Statistics Since the population size was small and the distribution of samples was non-normal, Mann-Whitney's non-parametric test was used to compare the differences of endogenous opioids in the brain tissue, microdialysis perfusate, and plasma of non-hibernating and hibernating Columbian ground squirrels.

Chemicals Met-enkephalin, Leu-enkephalin, DADLE, bestatin, aprotinin, and captropoil were purchased from Sigma. Dynorphin A (porcine) RIA kit and β-endorphin (rat) RIA kits were obtained from Peninsula Inc.

Results

Recovery of opioid peptides after solid phase extraction

Following SPE, the recovery rate of met-enkephalin was 61.50 ± 5.41% (n=3) and of leu-enkephalin, 87.33 ± 2.88% (n=3). The recovery rate for dynorphin A 1-13 was 59.33 ± 5.41% (n=3) and for β-endorphin, 71.98 ± 0.94% (n=3). These recovery rates were used in adjusting later calculations of endogenous opioid contents in various brain regions and plasma samples.

Changes in endogenous met-enkephalin and leu-enkephalin in several regions of the CNS of hibernating and non-hibernating brain homogenate samples

Figure V.1a shows the HPLC profile of standard met-enkephalin (1 ng), leu-enkephalin (1 ng), and DADLE (0.5 ng). The retention times for met-enkephalin, leu-enkephalin, and DADLE (external standard) were 4.2, 5.8, and 10.6 minutes, respectively (Figure V.1a). The sensitivity, defined as a minimum signal/noise ratio of 2 of our detection system was about 100 pg for met-enkephalin and about 150 pg for leu-enkephalin. Figure V.1b shows the HPLC chromatogram of a spiked (with 1 ng met-enkephalin, 1 ng leu-enkephalin, and 0.5 ng DADLE) brain homogenized sample and figure V.1c presents the same sample without the inclusion of spiked standards.

Met- and leu-enkephalin contents in several brain regions of the non-hibernating and hibernating Columbian ground squirrels were summarized in Table V.1. The amount of met-enkephalin in the hypothalamus and septum of the hibernating ground squirrels increased significantly (335% and 120% respectively) comparing to the non-hibernating animals. In the medulla region, on the other hand, met-enkephalin content declined significantly (57%) during hibernation. The total met-enkephalin concentration of all investigated regions combined was not significantly different between the non-hibernating and hibernating ground squirrels (P=0.20). As compared with met-enkephalin, leu-

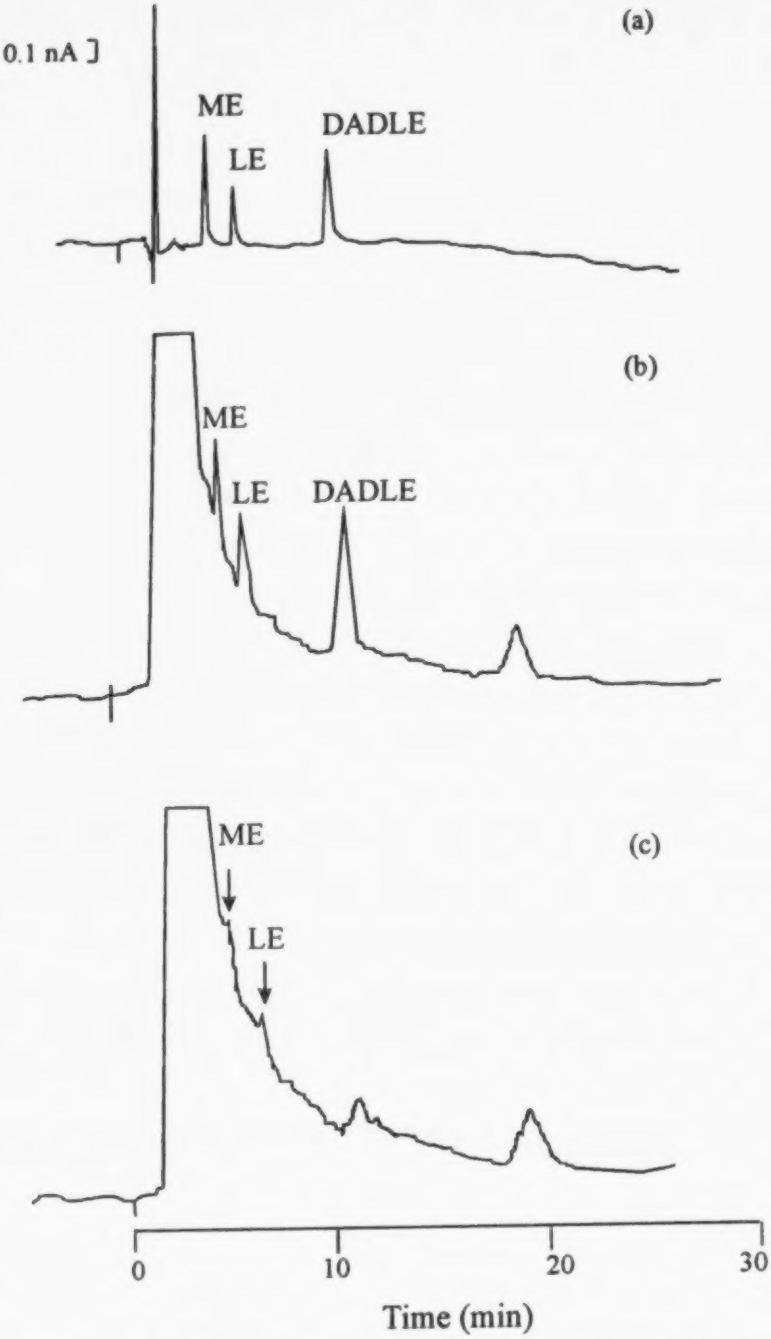

Figure V.1 HPLC chromatogram in the quantitive measurement of met-enkephalin and leu-enkephalin. (a) standard met-enkephalin (ME, 1 ng), leu-enkephalin (LE, 1 ng), and external standard DADLE (0.5 ng); (b) brain extract spiked with standard ME (1 ng), LE (1 ng), and DADLE (0.5 ng); (c) the same brain extract alone.

Table V.1 The amount of met-enkephalin and leu-enkephalin in the brain of non-hibernating and hibernating Columbian ground squirrels

	Non-hibernating (n=8)	Hibernating (n=6)	Mann-Whitney tests P Value
	Met-enkephalin (pmol/mg protein)		
Cortex	2.65 ± 0.72	4.10 ± 1.58	1.00
Hippocampus	8.51 ± 2.41	11.67 ± 2.73	0.52
Hypothalamus	21.16 ± 5.93	92.12 ±15.45	<0.01*
Medulla	10.99 ± 2.58	4.69 ± 0.82	0.04*
Septum	18.17 ± 2.31	40.10 ±10.22	0.05*
Striatum	67.25 ±14.56	63.14 ±14.88	0.81
	Leu-enkephalin (pmol/mg protein)		
Cortex	0.99 ± 0.35	0.90 ± 0.27	0.84
Hippocampus	1.59 ± 0.46	3.73 ± 1.12	0.15
Hypothalamus	5.36 ± 2.01	13.36 ± 1.91	0.01*
Medulla	1.34 ± 0.40	2.07 ± 1.27	0.47
Septum	3.32 ± 0.78	2.78 ± 0.29	0.68
Striatum	8.59 ± 1.94	8.93 ± 1.60	0.57

The results are expressed as mean ± s.e.. * indicates significantly different between non-hibernating and hibernating ground squirrels.

enkephalin was lower in overall concentration in both non-hibernating and hibernating squirrels. Except for a significant elevation (149%) in the hypothalamus and a slightly but insignificantly decreased (16%) in the septum, leu-enkephalin level during hibernation in most of the investigated regions was unaltered. Similar to met-enkephalin, the total concentration of leu-enkephalin of all investigated regions combined did not change significantly during hibernation (P=0.61).

Change in endogenous dynorphin A- and β-endorphin-like peptides in the central nervous system

Dynorphin A and β-endorphin contents were determined using commercial RIA kits. The total specific binding of the Dynorphin A 1-13 RIA kit was 32.31% with an ED_{50} (at which 50% of ^{125}I-Dynorphin A was displaced by standard dynorphin A from the anti-serum) of 3.93 pg/tube. The sensitivity (ED_{50}) of the β-endorphin RIA kit was 13.91 pg/tube with a maximal specific binding of 33.82%.

During hibernation, dynorphin A-like peptides in the hippocampus almost doubled the amount of non-hibernating Columbian ground squirrels (Table V.2). There was also a significant increase (56%) in dynorphin A-like peptides in the claustrum. The dynorphin A-immunoreactivity in the septum and hypothalamus of hibernating animals was also elevated slightly, though not significantly, by 58% and 46%, respectively. The total amount of dynorphin A immunoreactivity of the examined regions increased slightly, but not significantly, during hibernation (P=0.1). This increase was brought about by a modest elevation of dynorphin A-like peptides in most of the examined regions except the cerebellum, which showed a 61% decrease, though not significant, in dynorphin A immunoreactivity in the hibernating squirrels (Table V.2). During hibernation, β-endorphin immunoreactivity increased in the hypothalamus (165%), whereas it decreased to 21% in the medulla (Table V.3). The overall β-endorphin-like

Table V.2 Dynorphin A-like opioid content (fmol/mg protein) in the brain of Non-hibernating and hibernating Columbian ground squirrels

	Non-hibernating (n=8)	Hibernating (n=6)	Mann-Whitney tests P Value
Cerebellum	7.53 ± 1.83	2.90 ± 1.11	0.14
Claustrum	624.55 ±129.47	972.57 ±115.03	0.05*
Cortex	12.04 ± 4.05	15.68 ± 5.78	1.00
Hippocampus	41.44 ± 7.24	80.21 ± 21.26	0.04*
Hypothalamus	151.83 ± 54.69	221.80 ± 47.25	0.12
Medulla	30.23 ± 9.50	37.61 ± 12.08	0.75
Septum	91.46 ± 21.96	144.93 ± 35.50	0.14
Striatum	31.19 ± 11.63	94.65 ± 31.98	0.25

Each value represents mean ± s.e.. * indicates significantly different between non-hibernating and hibernating ground squirrels.

Table V.3 β-endorphin-like peptide content (fmol/mg protein) in the brain of Non-hibernating and hibernating Columbian ground squirrels

	Non-hibernating (n=8)	Hibernating (n=6)	Mann-Whitney tests P Value
Cerebellum	0.71 ± 0.23	0.76 ± 0.21	0.86
Claustrum	33.36 ±14.08	22.59 ±13.36	0.51
Cortex	1.20 ± 0.26	2.08 ± 0.64	0.42
Hippocampus	1.74 ± 0.47	1.71 ± 0.65	0.61
Hypothalamus	36.34 ± 7.03	60.03 ± 9.26	0.02*
Medulla	7.27 ± 3.15	1.54 ± 0.30	0.04*
Septum	32.77 ± 4.11	39.84 ±10.96	1.00
Striatum	6.95 ± 1.61	10.45 ± 4.04	0.70

The results present as mean ± s.e.. * indicates significantly different between non-hibernating and hibernating ground squirrels.

peptide in the investigated brain regions of hibernating squirrels did not differ (P=0.50) from that of non-hibernating squirrels.

Measurement of extracellular apparent turnover of dynorphin A and β-endorphin in the septum of non-hibernating and hibernating squirrel using microdialysis

At 37°C, the relative recovery rates of dynorphin A and β-endorphin were concentration independent. With the increase in flow rate, the relative recovery rates of both dynorphin A and β-endorphin decreased (Figure V.2). However, the absolute recovery increased when flow rate increased from 0.5 μl/min to 2 μl/min and gradually levelled off when flow rate reached 4 μl/min. At 2 μl/minute, the relative recovery was 2.75 ± 0.26% for dynorphin A (n=3) and 2.24 ± 0.09% for β-endorphin (n=3). The absolute recovery rate of dynorphin A was 0.110 fmol/min and that of β-endorphin was 0.090 fmol/min in a medium concentration of 2 nM dynorphin A and β-endorphin. At 5°C, because of the temperature effect, the recovery rates decreased. To compensate for the decline in recovery at 5°C, flow rate was reduced to half of that used at 37°C, i.e 1 μl/min. In this condition, the relative recovery rate of dynorphin A was 2.63 ± 0.24% (n=3) and that of β-endorphin was 1.93 ± 0.05% (n=3); these values were very close to their relative recovery rate at 37°C at a flow rate of 2 μl/min. The absolute recovery rate, in this condition, was 0.053 fmol/min for dynorphin A and 0.039 fmol/min for β-endorphin in the medium concentration of dynrophin A and β-endorphin as used at 37°C. Therefore, the absolute recoveries at 5°C used in the present experiment were about half of that at 37°C for both dynorphin A and β-endorphin because of temperature (Q_{10}) effect.

The apparent turnover of dynorphin A in the septal dialysate of non-hibernating ground squirrels (Tbr=37±1°C) was 0.081 ± 0.012 fmol/min (n=18). With the pre-determined recovery rate, the estimated interstitial concentration of dynorphin A-like peptides in the septum of non-hibernating Columbian ground squirrels was 1.47 ± 0.22 nM. During hibernation (Tbr=4-5°C), dynorphin A

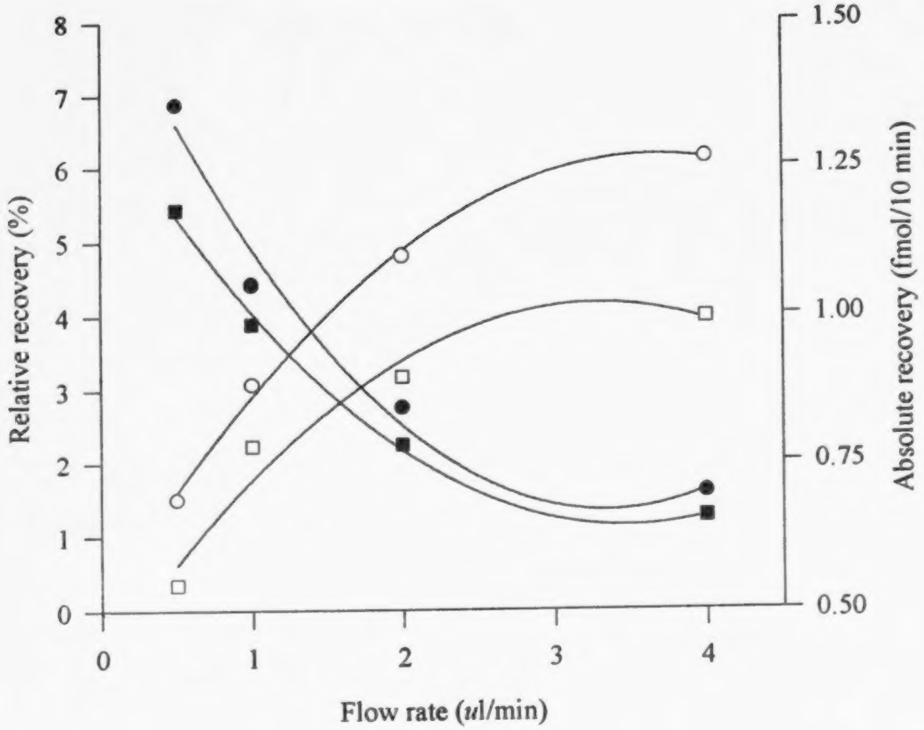

Figure V.2 Relative (closed symbol) and absolute (open symbol) recovery rates of Dynorphin A (circle) and β-endorphin (square) in the dialysate at 37°C.

apparent turnover in the perfusate was almost the same (0.080 ± 0.017 fmol/min) as that of non-hibernating animals. The estimated extracellular concentration, during hibernation, was 3.05 ± 0.64 nM, with an increase of 107% (n=7, P<0.01, Figure V.3). Following complete arousal (Tbr=37±1°C) from hibernation, dynorphin A apparent turnover increased to 0.134 ± 0.039 fmol/min (n=3) with an estimated extracellular concentration of 2.43 ± 0.71 nM, about 65% higher than the non-hibernating level. The inter-bout euthermic extracellular dynorphin A immunoreactivity was, therefore, between the hibernating and non-hibernating state, and not significantly different from either of them (P=0.33 for non-hibernating vs. inter-bout euthermia and P=0.40 for hibernating vs. inter-bout euthermia).

The β-endorphin apparent turnover in the septal perfusate of non-hibernating ground squirrels was 0.089 ± 0.017 fmol/min (n=18, 18 samples from 5 animals) and, thus, an extracellular concentration of 1.99 ± 0.38 nM. During hibernation, the apparent turnover of β-endorphin decreased slightly to 0.073 ± 0.028 fmol/min (n=7, 7 samples from 3 animals), but its estimated interstitial concentration increased (90%) significantly to 3.78 ± 1.44 nM (P=0.01, Figure V.3). When animals were completely aroused from hibernation, there was a drastic increase in β-endorphin apparent turnover (97%, 0.175 ± 0.035 fmol/min, n=3, 3 samples from 3 animals) which led to an even higher extracellular concentration of 3.90 ± 0.78 nM. Thus, the extracellular concentration of β-endorphin-like peptide in the inter-bout euthermia was significantly higher (P=0.02) than that of squirrels in the non-hibernating state.

Changes in peripheral opioid concentration

The met-enkephalin concentration in the plasma of hibernating Columbian ground squirrels was significantly higher (38%) than that of non-hibernating animals (n=5, P<0.01). However, the concentration of leu-enkephalin did not change in the hibernating state (0.12 ± 0.01 pmol/ml, Figure V.4) as compared

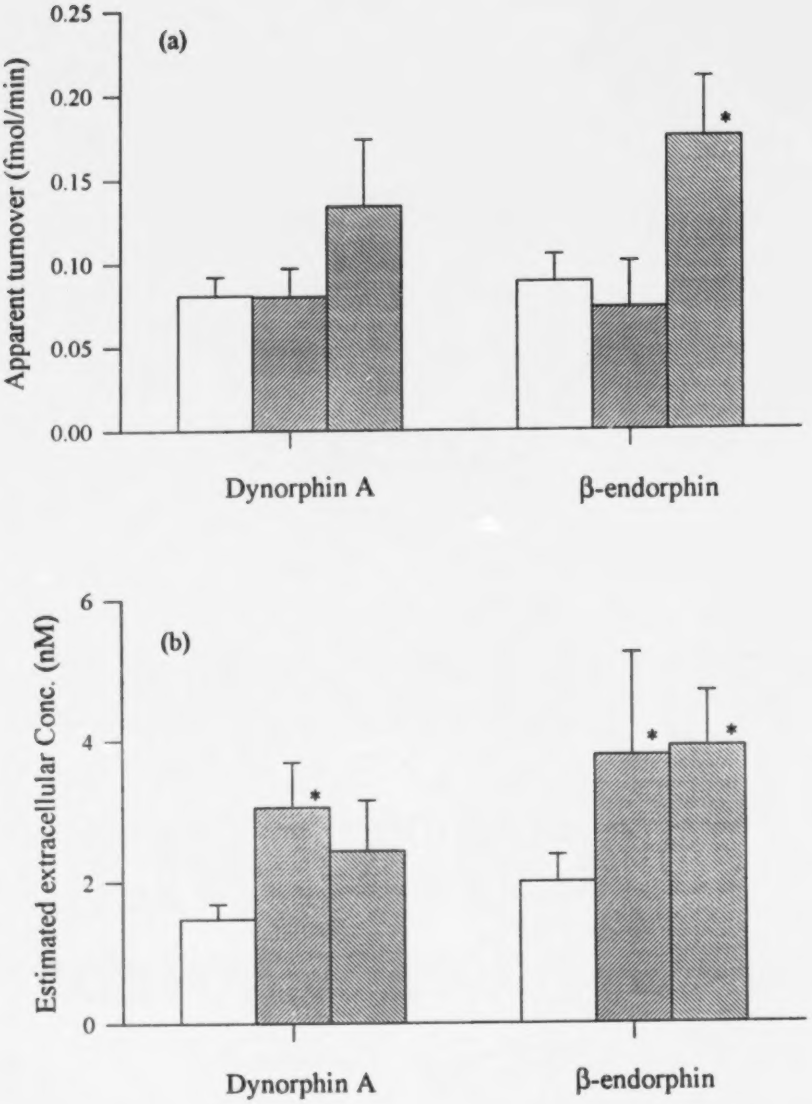

Figure V.3 Apparent turnover (a) and estimated extracellular concentrations (b) of dynorphin A- and β-endorphin-like-immunoreactivity in the septum of non-hibernating (clear bar, n=18), hibernating (left hatched bar, n=7), and inter-bout euthermic (right hatched bar, n=3) Columbian ground squillres. * indicates significantly different from the non-hibernating animals (P<0.05).

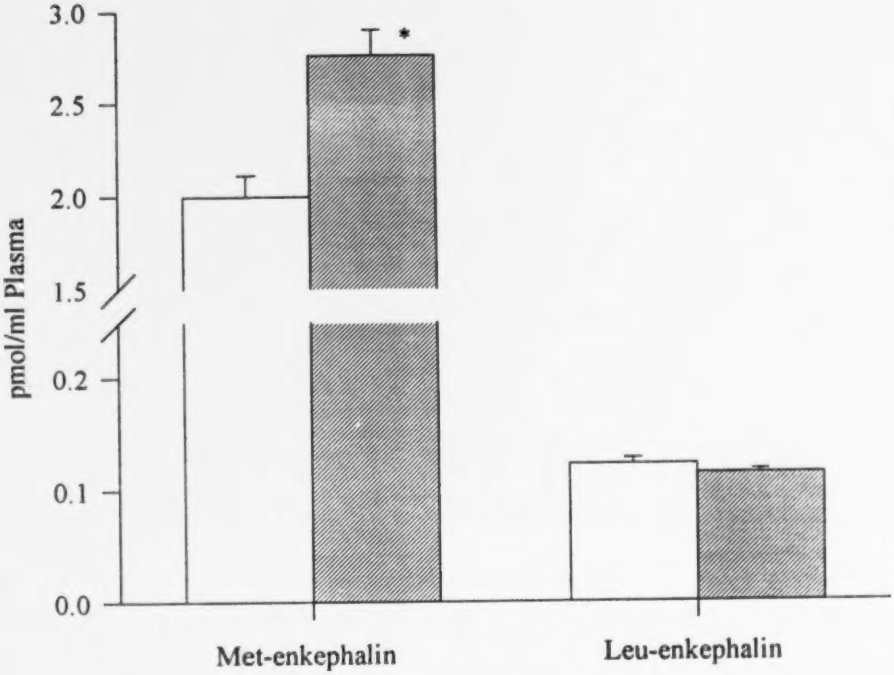

Figure V.4 Met-enkephalin and leu-enkephalin concentrations in the plasma of non-hibernating (open bar, n=5) and hibernating (hatched bar, n=5) Columbian ground squirrels. * indicates significantly different from the non-hibernating animals ($P<0.05$).

to the non-hibernating animals (0.11 ± 0.01 pmol/ml, n=5, P=0.21). Similar to met-enkephalin, there was a drastic elevation (401%) of dynorphin A immunoreactivity in the plasma of hibernating ground squirrels (n=4) in comparison to non-hibernating animals (n=9, P=0.02, Figure V.5). In contrast, β-endorphin concentration in the plasma decreased by 29% during hibernation (n=4) as compared to the non-hibernating animals (n=9, P=0.25, Figure V.5).

Discussion

The present study demonstrated a state-dependent increase in endogenous opioid peptides. However, this was not an overall elevation of opioid content in every CNS region. Rather, the increase was only limited to a few regions, such as the hippocampus, septum, and hypothalamus. In contrast, there was even a decrease in opioid contents in other regions (mainly medulla). It has been shown that met-enkephalin, leu-enkephalin, β-endorphin, and dynorphin are produced from three distinct genes via different pathways (Brush and Shain 1989, Simon 1991) and each of them distributes differently in the brain and in the periphery (Cuello 1983, Hokfelt et al. 1984). Changes in opioid content in the CNS and peripheral systems have been demonstrated to be related to physiological or pathological responses (Kurumaji et al. 1987, Alessio et al. 1989, Nabeshima et al. 1992). Therefore, the diversity of changes in endogenous opioids in various CNS regions and in the peripheral during hibernating may be related to the state of hibernation.

Our observed increases in endogenous opioid contents during hibernation were not due to cold or other physical stresses. The patterns of change in brain opioid peptides under various stresses have been revealed. For instance, cold and immobilization caused a decrease in met-enkephalin-like immunoreactivity in the prefrontal cortex, hypothalamus, thalamus, hippocampus, and striatum

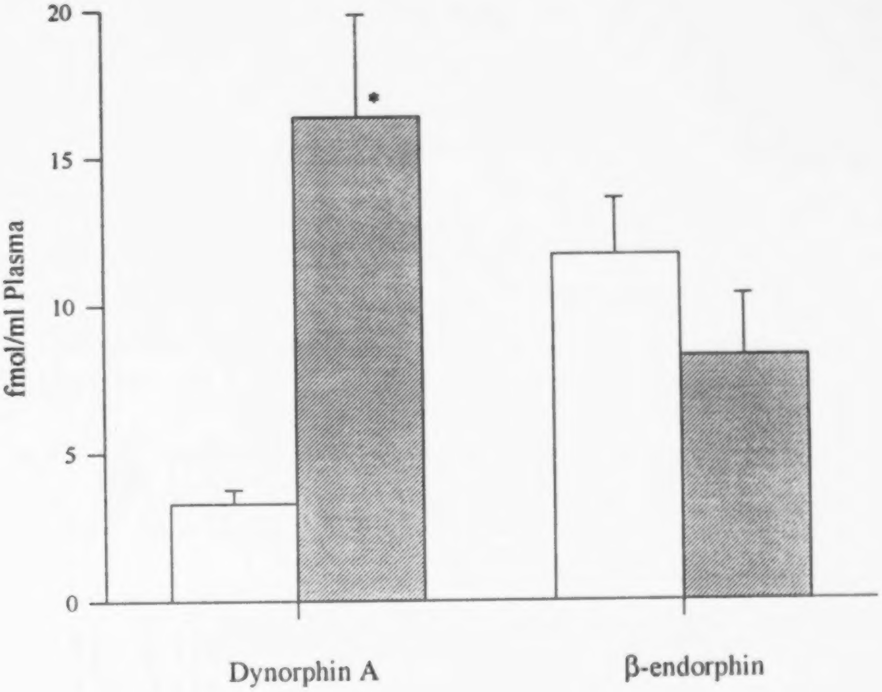

Figure V.5 Concentrations of dynorphin A- and β-endorphin-like peptides in plasma of non-hibernating (clear bar, n=9) and hibernating (filled bar, n=4) ground squirrels. * indicates significantly different from non-hibernating squirrels ($P<0.05$).

(Kurumaji et al. 1987). Further, both environment-induced conditioned stress and forced swimming reduced concentrations of met-enkephalin in the striatum and leu-enkephalin in the hypothalamus, but induced an increase in dynorphin A concentration in the hypothalamus (Nabeshima et al. 1992). During hibernation, both met-enkephalin and leu-enkephalin concentrations increased significantly in the hypothalamus, which was opposite to stress-induced decrease in endogenous opioids (Kurumaji et al. 1987). The significant elevation of septal met-enkephalin level during hibernation was also different from the unchanged levels during cold and immobilization stress (Kurumaji et al. 1987). The 203% increase in dynorphin A content in the striatum during hibernation is also different from the decrease in dynorphin content under stress (Nabeshima et al. 1992). These state-dependent changes echo our previous findings that an increase in met-enkephalin immunocytochemical activity in the lateral septum only appeared in the hibernating ground squirrels but not in the artificially induced hypothermic ground squirrels while in the non-hibernating phase (Nurnberger et al. 1991). In addition, the increase in opioid activity was not uniformly expressed in the CNS. A significant reduction in medulla met-enkephalin and β-endorphin content has been observed. Likewise, the activity changes of the four investigated endogenous opioids were not identical to each other in different hibernation states.

One of the interesting observations was that dynorphin A-like peptides were extremely high in the claustrum (CLA). During hibernation, dynorphin A-immunoreactivity in this region was much higher than the non-hibernating state. The claustrum has been speculated to be involved in feeding (Simpson and Fitch 1988). A decrease in 5-HT receptor binding in the claustrum was found to be associated with a reduction in food intake in chronic 5-HT$_1$ agonist-treated animals (Rouru et al. 1993). The κ opioid agonists have been shown to modulate food intake (Stanley et al. 1989). In addition, activation of κ receptors has been demonstrated to stimulate fat intake (Romsos et al. 1987). Increase in dynorphin immunoreactivity in the cortex, hypothalamus, and striatum has been shown in the hypophagic phase of the hibernation cycle (Nizielski et al. 1986). Thus, our observed increase in dynorphin A-like activity in the CLA during hibernation may be related to the modulation of food intake at different hibernation states, such as fall fattening and the ensuing anorexia prior to hibernation. However, the precise roles of the opioids in the CLA on hibernation and other physiological events still require further investigation.

Microdialysis has been shown to be a very useful tool in investigating small and yet relatively slow changes of neurotransmitters in the extracellular space during behavioral or pharmacological manipulation (Westerink and Justice 1991). Different from most neurotransmitters, opioids have not been shown to have a reuptake mechanism by neuronal terminals. Therefore, the interstitial concentration of endogenous opioid peptides reflects an equilibrium, among the rates of release, degradation, and diffusion. From this point of view, the apparent turnover rate (which is the amount of opioids diffused into the dialysate per unit time) may be more appropriate an index in representing opioid activity in different hibernating states. However, because extracellular concentration is most commonly used in presenting microdialysis data in publications, the estimated extracellular opioid concentrations in different hibernation states were also calculated in the present study. During hibernation, because of the over 30°C drop in Tb, the enzymic, neuronal, physiological, and metabolic activities all decreased significantly (for review see Wang 1988, 1989). The lowering in Tb also reduced diffusion rate. It is, therefore, expected that opioid turnover would decrease correspondingly during hibernation. On the contrary, our observation showed that the apparent turnover of dynorphin A-like peptides in the septum of hibernating squirrels (Tbr=5°C) was about the same as that of the non-hibernating animals at Tbr of 37°C. Based on an assumed Q_{10} of 2.5 for biological systems, a 30°C difference would entail a 16 fold difference in biological rate functions. Thus, it may be implied that during hibernation, the apparent turnover of dynorphin A was 15 times greater than that seen in the euthermic, non-hibernating state. The septal turnover rate of β-endorphin during hibernation was slightly lower than the non-hibernating animals. However, a 30°C decrease in Tb during hibernation would have brought about a 94% decrease in biological rate functions, assuming a Q_{10} of 2.5. Our observed 12% decrease in β-endorphin apparent turnover may indicate an extremely high activity during hibernation. Thus, unlike other suppressed physiological parameters, the very high activities of dynorphin A and β-endorphin during hibernation may suggest their functional roles in hibernation. In addition, the changes in dynorphin A and β-endorphin turnover in the septum were not following the same pattern after arousal. Dynorphin A turnover was increased slightly (not significant) in inter-bout euthermia compared with non-hibernation state whereas β-endorphin turnover increased drastically after arousal from hibernation. This may imply different roles of these two endogenous opioids in regulating hibernation.

In terms of extracellular concentration, increased dynorphin A-like peptides in the lateral septum of hibernating ground squirrels was in agreement with brain tissue homogenate results although the increase indicated in brain homogenates was less profound. This may be caused by the large size of tissue sample used which combined several nuclei and changes in only one or a few nuclei may be masked by other nuclei without changes. However, an elevation in septal interstitial β-endorphin concentration, observed in microdialysis, was not evident in the tissue homogenate sample. Other than the above stated reason, another possibility for this discrepancy is that opioid content in homogenates includes both intracellular and extracellular peptides. Intracellular opioids are those being translated and stored. Only extracellular opioids, which are released to the interstitial space, are functionally involved and may not necessarily correlate with their intracellular levels. Therefore, concentration and apparent turnover measured by *in vivo* microdialysis seem to be better indicators of functional neuronal activity.

Peripheral opioids are synthesized from different sources than are CNS opioids (Zakarian and Smyth 1982, Hokfelt et al. 1984, Akil et al. 1988). Plasma β-endorphin is mainly from the pituitary whereas enkephalins and dynorphin are from adrenal medulla and/or gastrointestinal tract (Zakarian and Smyth 1982, Hokfelt et al. 1984, Negri et al. 1989). The regulation of peripheral opioids is also different from the central system (Hokfelt et al. 1984, Genazzani et al. 1984, Vescovi and Coiro 1993) and changes differently in response to various physiological or pathological conditions (Alessio et al. 1989). Physiologically, peripheral administration of dynorphin or other κ agonists has been shown to induce hypothermia (Adler et al. 1991) and reduce heart rate and cardiac contractility (Illes 1989). Chronic administration of κ agonists also depresses the hypothalamic-pituitary-adrenocortical (HPA) activity and decreases plasma β-endorphin level (Milanes et al. 1991). The observed increase in dynorphin A-immunoreactivity in the plasma of hibernating animals may thus be speculated to have exerted a facilitatory role for hibernation by activating peripheral κ receptors. The peripheral β-endorphin, on the other hand, has been suggested to modulate the activity of other neurohormones, stimulate HPA activity, and stimulate lipolysis (Richter et al. 1983, Dalayeun et al. 1993, Vescovi and Coiro 1993). Our observed decrease in β-endorphin-like peptides in the plasma of hibernating ground squirrels is consistent with the reported generally depressed endocrinological functions during hibernation (Wang 1982), including that of the HPA. However, the precise functions of these opioids in regulating hibernation still needs to be further investigated.

The increase in endogenous opioids during hibernation could be a result of an increase in gene expression. Opioid gene expression has been shown to be regulated by neuronal activity and/or chronic exposure to opioid ligands (L'Hereault and Barden 1991, Romualdi et al. 1991, Comb et al. 1992, McMurray et al. 1992, Pelletier 1993, Yebenes and Pelletier 1993). The persistence of neuronal activities in the septum, sensory cortex, and medial preoptic area during hibernation (Strumwasser 1959) may have contributed to the increase of opioids activity during hibernation. However, a reduction in opioid degradation at low Tb can not be completely excluded. Carrier molecules for enkephalins have been shown to exist in plasma and they can increase the stability of plasma enkephalins (Roda et al. 1983). Experiments have also shown the existence of endogenous plasma enkephalinase inhibitors which protect opioids from being degraded (Marini et al. 1990). The discovery of an increase in α_2-macrogolbulin, a broad-spectrum plasma protease inhibitor, during hibernation (Srere et al. 1992, Martin et al. 1993) further supports the possibility of reduced degradation of macromolecules during hibernation. Further experiments on opioid gene expression during hibernation are thus needed to decipher the alteration of endogenous opioid metabolism during hibernation as well as their functional roles in regulating hibernation.

References

Adler M.W., Bradley E., Martinez R., and Geller E.B. (1991) Production of hypothermia in the guinea pig by a kappa-agonist opioid alone and in combination with chlorpromazine. Pharmacol Biochem Behav 40:129-132

Akil H., Watson S. T., Yang E., Lewis M. E., Khaehaturian H. and Waller J. M. (1984) Endogenous opioids: biology and function. Ann Rev Neurosci 7:223-255

Akil H., Bronstein D., and Mansour A. (1988) Overview of the endogenous opioid systems: anatomical, biochemical and functional issues. Endorphins, Opiates and Behavioral Processes Rodgers R.J. and Cooper S.J. (eds), pp 1-23, Chichester, New York.

Alessio L., Salar F., Battaini F., Govoni S., and Trabucchi M. (1989) Endogenous opioids in human biological fluids: in search of a functional role. Opioid Peptides in Biological Fluids Genazzani A.R. and Negri M. (eds), pp 45-52, Parthenon, Lancs.

Beckman A. L., Llados-Eckman C., Stanton T. L., and Adler M. W. (1981) Physical dependence on morphine fails to develop during the hibernating state. Science 212:527-529

Beckman A., Dean R. R.,and Wamsley J. K. (1986) Hippocampal and cortical opioid receptor binding: changes related to the hibernation state. Brain Res 386:223-231

Benvensite H. (1989) Brain moicrodialysis. J Neurochem 52:1667-1679

Bradford M.M. (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 27:248-254

Brush F.R. and Shain C.N. (1989) Endogenous opioids and behavior. Psychoendocrinology Brush F.R. and Levine S. (eds), pp 379-435, Academic Press, San Diago.

Burks T.F. (1991) Opioids and opioid receptors in thermoregulation. Thermoregulation: Pathology, Pharmacology and Therapy Schonbaum E. and Lomax P. (eds), pp 489-508, Pergmon Press, New York.

Civelli O., Douglass J., Rosen H., Martens G., and Herbert E. (1986) Biosynthesis of opioid peptides. Opioid Peptides: Molecular Pharmacology, Biosynthesis, and Analysis. NIDA Monograph Series 70:21-42, NIDA publicantion, Rockville.

Comb M.J., Kobierski L., Chu H.M., and Tan Y. (1992) Regulation of opioid gene expression: a model to understand neural plasticity. Molecular Approaches to Drug Abuse Research Volume II: Structure, Function, and Expression. NIDA Research Monograph Vol 126. Lee T.N.H. (ed), pp 98-112, NIDA publication, Rockville.

Cuello A.C. (1983) Central distribution of opioid peptides. Medical Bull 39:11-2

Dalayeun J.F., Nores J.M., and Bergal S. (1993) Physiology of β-endorphins. a close-up veiw and a review of the literature. Biomed & Pharmacother 47:311-320

Genazzani A.R., Petraglia F., Nappi G., Martignoni E., Leo M.D., and Facchinetti F. (1984) Endorphins in peripheral plasma: origin and influencing factors. Central and Peripheral Endorphins: Basic and Clinical Aspects Muller

E.E. and Genazzani A.R. (eds), pp 89-98, Raven Press, New York.

Genazzani A.R., Romano G., Brilli G., and Petraglia F. (1989) Neuroendocrine control of plasma bete-endorphin sercetion. Opioid Peptides in Biological Fluids Genazzani A.R. and Negri M. (eds), pp 11-16, Parthenon, Lancs.

Gosnell B.A. (1987) Central structures involved in opioid-induced feeding. Fed Pro 46:163-167

Handler C.M., Geller E.B., and Adler M.W. (1992) Effect of m-, k-, and d-selective opioid agonists on thermoregulation in the rat. Pharmacol Biochem & Behav 43:1209-1216

Hokfelt T., Vincent S.R., Dalsgaard C.J., Herrera-Marschitz M., Ungerstedt U., Schultzberg M., Chiristensson I., and Terenius L. (1984) Some aspects on distribution and role of opioid peptides in the central and peripheral nervous system. Central and Peripheral Endorphins: Basic and Clinical Aspects Muller E.E. and Genazzani A.R. (eds), pp 1-16, Raven Press, New York.

Hughes J., Simth T., Kosterlitz H.W., Fothergill L.A., Morgan B., and Morris H.R. (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 258:577-579

Illes P. (1989) Modulation of transmitter and hormone release by multiple neuronal opioid receptors. Rev Physiol Biochem Pharmacol 112:141-233

Jackisch R., Geppert M., Lupp A., Huang H.Y., and Illes P. (1988) Types of opioid receptors modulating neurotransmitter release in discrete regions. Regulatory Roles of Opioid Peptides Illes P. and Farsang C. (eds), pp 240-258, VCH, Weinheim.

Kenderick K.M. (1989) Use of microdialysis in neuroendocrinology. Methods in Enzymology 168:182-205

Khachaturian H., Lewis M.E., Schafer M.K.-H., and Watson S.J. (1985) Anatomy of the CNS opioid system. TINS 8:111-119

Kramarova L.I., Kolaeva S.G., Yukhanaov R.Y., and Rozhanets V.V. (1983) Content of DSIP, enkephalins, and ACTH in some tissue of active and hibernating ground squirrels. Comp Biochem Physiol 74C:31-33

Kunos G., Mosqueda-Garcia R., and Mastrianni J. A. (1988) Endophinergic neurons in the brainstem and the control of blood pressure and heart rate.

Regulatory Roles of Opioid Peptides Illes P. and Farsang C. (eds), pp 460-470, VCH, Weinheim.

Kurumaji A., Takashima M., and Shibuya H. (1987) Cold and immobilization stress-induced changes in pain responsiveness and brain met-enkephalin-like immunoreactivity in the rat. Peptides 8:355-359

Lee T. F., Nurnberger F., Jourdan M. L. and Wang L. C. H. (1989) Possible involvement of septum in seasonal changes in thermoregulatory responses to met-enkephalinamide in ground squirrels. Thermoregulation: Research and Clinical Applications Schonbaum E. and Lomax P. (eds), pp 200-203, Kruger, Basel.

L'Hereault S. and Bardeb N. (1991) Regulation of proopiomelanocortin messenger RNA concentrations by opioid peptides in primary cell cultures of rat hypothalamus. Mol Brain Res 10:115-121

Marini M., Roscetti G., Bonginrno L., Urbani A., and Roda L.G.(1990) Hydrolysis and protection from hydrolysis of enkephalins in human plasma. Neurochem Res 15:61-67

Martin S.L., Srere H.K., Belke D., Wang L.C.H., and Carey H.V. (1993) Differential gene expression in the liver during hibernation in ground squirrels. Life in the Cold: Ecological, Physiological, and Molecular Mechanisms. Cary C., Florant G.L., Wunder B.A., and Horwitz B. (eds), pp 443-454, Westview Press, Colorado.

McMurray C. T., Pollock K. M., and Douglass I. (1992) Cellular and molecular anaylsis of opioid peptide gene expression. Molecular Approcahes to Drug Abuse Research Volume II: Structure, Function, and Expression. NIDA Research Monograph Vol 126. Lee T.N.H. (ed), pp 113-131, NIDA publicantion, Rockville.

Milanes M.V., Gonzalvez M.L., Fuente T., and Vargas M.L. (1991) Pituitary-adrenocortical response to acute and chronic addministration of U-50,488H in the rat. Neuropeptides 20:95-102

Nabeshima T., Katoh A., Wada M., and Kameyama T. (1992) Stress-induced changes in brain met-enkephalin, leu-enkephalin and dynorphin concentrations. Life Sci 51:211-217

Negri M., Tonnarini G., Mariani R., Martino R., and Locuratolo N. (1989) Plasma met-enkephalin in physiopathological conditions. Opioid peptides in Biological Fluids. Genazzani A.R. and Negri M. (eds), pp 185-196, Parthenon, Lancs.

Nizielski S.E., Levine A.S., Morley J.E., Hall K.A., and Gosnell B.A. (1986) Seasonal variation in opioid modulation of feeding in the 13-lined ground squirrel. Physiol Behav 37:5-9

Nurnberger F, Lee T. F., Jourdan M. L., and Wang L. C. H. (1991) Seasonal changes in methionine-enkephalin immunoreactivity in the brain of hibernator, *Spermophilus columbianus*. Brain Res 547:115-121

Olson G.A., Olson R.D., and Kastin A.J. (1990) Endogenous opiates: 1989. Peptides 11:1277-1304

Olson G.A., Olson R.D., and Kastin A.J. (1992) Endogenous opiates: 1991. Peptides 13:1247-1287

Pelletier G. (1993) Regulation of proopiomelanocortin gene expression in rat brain and pituitary as studied by *in situ* hybridization. Annals New York Acad Sci 683:246-259

Rabkin S.W. (1989) Cardiorespiratory effects of D-Ala^2-Me-Phe-4-Met-(o)-ol enkephalin in the third ventricle and in anteriorhypothalamic and paraventricular areas of the rat brain. Cardiovasc Res 23:904-912

Richter W.O., Kerscher P., and Schwandt P. (1983) β-endorphin stimulates in vivo lipolysis in the rabbit. Life Sci 33(suppl):743-746

Roda L.G., Marco V.D., and Possenti R. (1983) Stability of peripheral enkephalins. Degradation of Endogenous Opioids: Its Relevance in Human Pathology and Therapy Ehrenpreis S. and Sicuteri F. (eds), pp , Raven Press, New York.

Romsos D.R., Gosnell B.A., Morley J.E., and Levine A.S. (1987) Effects of kappa opiate agonists, cholecystokinin and bombesin on intake of diets varying in carbohydrate- to -fat ratio in rats. J Nutrition 117:976-985

Romualdi P., Lesa G., and Ferri S. (1991) Chronic opiate agonists down-regulate prodynorphin gene expression in rat brain. Brain Res 563:132-136

Rouru J., Pesonen U., Isaksson K., Huupponen R., and Koulu M. (1993) Effect of chronic treatment with TFMPP, a 5-HT$_1$ receptor agonist, on food intake, weight gain, plasma insulin and neuropeptides mRNA expression in obese Zucker rats. Eur J Pharmacol 234:191-198

Schick R. and Schusdziarra V. (1985) Physiological, pathophysiological and pharmacological aspects of exogenous and endogenous opiates. Clin Physiol Biochem 3:43-60

Simon E.J. (1991) Opioid receptors and endogenous opioid peptides. Med Res Rev 11:357-374

Simpson J. A. and Fitch W. (1988) Applied Neurophysiology Wright, London.

Spencer R.L., Hruby V.J, and Burks T.F. (1990) Alteration of thermoregulatory set point with opioid agonists. J Pharmacol Exp Ther 252:696-705

Srere H.K., Wang L.C.H., and Martin S.L. (1992) Central role for differential gene expression in mammalian hibernation. Proc Natl Acad Sci USA 89:7119-7123

Stanley B.G., Lanthier D., and Leibowitz S.H. (1989) Multiple brain sites sensitive to feeding stimulation by opioid agonists: a cannula-mapping study. Pharmacol Biochem Behav 31:825-832

Stengaard-Pedersen K. (1989) Opioid Peptides and Receptors Progress in Histochemistry and Cytochemicstry. Vol. 20, No. 3. G Fisher, Stuttgart.

Strumwasser F. (1959) Regulatory mechanisms, brain activity and behaviour during deep hibernation in the squirrel, *Citellus beecheyi*. Am J Physiol 196:23-30

Ungerstedt U. (1991) Introduction to intracerebral microdialysis. Microdialysis in the Neurosciences Robinson T.E. and Justice J.B. (eds), pp 3-22, Elsevier, Amsterdam.

Vescovi P.P. and Coiro V. (1993) Hyperthermia and endorphins. Biomed & Pharmacother 47:301-304

Westerink B.H.C. and Justice J.B.Jr. (1991) Microdialysis compared with other *in vivo* release models. Microdialysis in the Neurosciences Robinson T.E. and Justice J.B. (eds), pp 23-46, Elsevier, Amsterdam.

Wang L.C.H. (1982) Hibernation and the endocrine. Hibernation and Torpor in Mammals and Birds Lyman CP., Willis J.., Malan A., and Wang L.C.H. (eds), pp 2006-236, Academic Press, London.

Wang L. C. H., Lee T. F., and Jourdan M. L. (1987) Seasonal difference in thermoregulatory responses to opiates in a mammalian hibernator. Pharmacol Biochem Behav 26:565-571

Wang L.C.H. (1988) Mammalian hibernation: an escape from the cold. Advances in Comparative and Environmental Physiology Vol.2, Gilles R. (ed), pp 1-45, Springer-Verlag, Berlin.

Wang L.C.H. (1989) Ecological, physiological, and biochemical aspects of torpor in mammals and birds. Advances in Comparative and Environmental Physiology Vol.4, Wang L.C.H. (ed), pp 361-401, Springer-Verlag, Berlin.

Yeadon M. and Kitchen I. (1989) Opioids and respiration. Prog Neurobiol 33:1-16

Yebenes E.G. and Pelletier G. (1993) Opioid regulation of proopiomelanocortin (POMC) gene expression in the rat brain as studied by *in situ* hybridization. Neuropeptides 25:91-94

Yim G.K.W. and Lowy M.T. (1984) Opioids, feeding, and anorexias. Federation Proc 43:2893-2897

Zakarian S. and Smyth D.G. (1982) β-endorphin is processed differently in specific regions of rat pituitary and brain. Nature 296:250-252

CHAPTER SIX

General discussion and conclusions

Mammalian hibernation is a complex phenomenon, involving a series of complicated adjustments at the cellular, biochemical, physiological, neuronal, and behavioral levels. Transition from one state of the hibernation cycle to the other is precisely controlled by an endogenous circannual rhythm located in the central nervous system (CNS). The regulating processes involved in a hibernation bout, though still largely unknown, comprise at least of neuronal network activation, neurochemical interactions, and receptor and ion channel activation, affecting cardiovascular, respiratory, and metabolic functions. Among the numerous neurochemicals, endogenous opioids have been speculated to be one of the important components in regulating hibernation. Previous investigations have suggested an increase in opioid activity during hibernation and antagonism of opioid activity by exogenous opioid antagonists induces premature arousal from hibernation (for review see Chapter One). Our observations in this thesis research demonstrate a hibernation state-dependent increase in various endogenous opioid activities and a correlated decrease in opioid receptor efficacy in several CNS regions. Furthermore, an elevation of plasma opioid peptides has also been observed during hibernation.

CNS control of hibernation

Mammalian hibernation is an active, precisely controlled process, much different from poikilothermic torpor. Prior to any visible transitions, when animals are about to enter into or arouse from hibernation, changes in neuronal electro-activity always precede physiological changes (Strumwasser 1959, Wunnenberg et al. 1978, Lyman 1982). This implies that the transition is a controlled event rather than passively manipulated by external cues. Even at very low Tb, hibernating animals are still able to control their autonomic physiological functions (Heller 1979, Beckman and Stanton 1982, Lyman 1982). Precise regulation of the re-adjusted autonomic activities is vital for survival of the animals under such depressed metabolic conditions. The hypothalamic region, along with the brain stem reticular formation (BSRF), are the primary regions for thermoregulation and autonomous physiological adjustments (Heller 1979, Cohen and Shermen 1983). The septo-hippocampal complex, as an important relay station between the hypothalamus and BSRF (for review see Chapter One), has been demonstrated to be involved in thermoregulation (Murakami et al. 1984, Boulant et al. 1989, Lee et al. 1989, Zeisberger 1990), food intake (King and Nance 1986, Stanley et al. 1989), and other autonomic functions (Wang and Ingenito 1992, 1994, Rector et al. 1993). These limbic regions still remain metabolically and electrically active during hibernation (Strumwasser 1959, Wunnenberger et al. 1978, Kilduff et al. 1986, 1989). It is, thus, hypothesized that an inhibitory influence from the septo-hippocampal complex to the BSRF induces the entry into hibernation (Heller 1979), whereas, reversal of this inhibition in the septal-hippocampal region causes arousal from hibernation (Heller 1979, Beckman and Stanton 1982, Belousov and Belousova 1992, Belousov 1993). Therefore, the investigations in the thesis are mainly focused on the septo-hippocampal regions.

Central distribution of endogenous opioids and opioid receptors in Columbian ground squirrels

All three types of opioid receptor, μ, δ, and κ, have been demonstrated to be widely distributed and especially dense in the limbic structure of the CNS (Mansour et al. 1988, 1994a, Simon 1991). Receptor autoradiography (Chapter Four) revealed that, among the investigated regions, the density of μ opioid receptor binding in non-hibernating ground squirrels is as follows: hippocampal fissure >> lateral septum > CA3 > preoptic area > CA1 = dentate gyrus > medial septum. This distribution pattern is different from that of rat brain, investigated both by receptor binding using autoradiography (Mansour et al.

1986, 1988, Sharif and Hughes 1989) and receptor mRNA using *in situ* hybridization (George et al. 1994, Mansour et al. 1994a, 1994b). The binding density of the δ receptor is hippocampal fissure > dentate gyrus > CA1 > CA3 > lateral septum > preoptic area > medial septum. The localization of δ receptors is also different from rat and mouse (Mansour et al. 1988, Garters et al. 1993, George et al. 1994). *Kappa* agonist binding, on the other hand, is different from μ and δ: Claustrum >> dentate gyrus > lateral septum = CA3 > preoptic area = CA1 > medial septum. Except for its high density in the claustrum of several species (DePaoli et al. 1994, Mansour et al. 1994c), localization of the κ receptor is also species-specific (Mansour et al. 1988, Sharif and Hughes 1989). Base on the measured receptor density (maximal binding) of μ, δ, and κ opioid receptors in the brain of non-hibernating Columbian ground squirrels (Chapter Four), their proportional make-up is about 41%, 29%, and 30%, respectively. This proportional make-up is also different from rats, mice, guinea pigs, pigeons, and humans (Mansour et al. 1988). The species differences in opioid receptor distribution pattern and overall ratio have long been in evidence (Herkenham and McLean 1988, Mansour et al. 1988, Sharif and Hughes 1989). Whether the species variation is related to specific physiological characteristics or adaptations of each species is currently uncertain.

Similar to opioid receptors, endogenous opioids, derived from proopiomelanocortin, proenkephalin, and prodynorphin families, have also been located throughout the CNS (Akil et al. 1984, 1988, Khachaturia et al. 1985, Mansour et al. 1986, 1988, Brush and Shain 1989). The concentration of β-endorphin, the endogenous opioid with both μ and δ potency, is high in the hypothalamus, septum, and claustrum regions, and low in the frontal cortex, hippocampus, medulla, striatum, and cerebellum of non-hibernating Columbian ground squirrels (Chapter Five). Overall, the concentrations of met-enkephalin and leu-enkephalin, endogenous ligands preferring the δ receptors, are about two orders of magnitude higher than other endogenous opioids. Their concentrations are high in the striatum, moderate in the hypothalamus and septum, and low in the hippocampus. The concentration of dynorphin A-related peptides is extremely high in the claustrum, correlating well with the dense distributions of κ receptors in the same area. The concentration of dynorphin A-like peptides is also high in the hypothalamus and septum, moderate in the hippocampus and striatum, and low in the frontal cortex and cerebellum. As indicated above, the distribution and density of opioid receptors and endogenous opioids are consistent for most of the investigated regions. However, because of the complexity and low specificity of endogenous opioid peptides, mismatches between the peptides and receptors do exist, as reported both in this study and in previous studies (Akil et al. 1984, Khachaturian et al. 1985, Mansour et al. 1988, 1994a, Reiner et al. 1989). This endogenous opioid-receptor mismatch is speculated to be a result of low specificity of endogenous opioids, diffusion of opioids in the synaptic space to reach their receptors in nearby regions, unidentified receptors, and "spare" receptors (Herkenham and McLean 1988, Mansour et al. 1988, 1994a, Reiner et al. 1989). The first two are most likely responsible for the mismatch in our observations.

Changes in endogenous opioid activity in the hibernation cycle

When receptors are being exposed to their putative agonist, either endogenously released or exogenously applied, for an extended period of time, the effectiveness of the ligand to induce corresponding physiological or pharmacological effects is reduced. This can be a result of receptor desensitization or receptor down-regulation. Desensitization is characterized as a reversible conformational change of the receptors without altering the receptor numbers (Johnston and Wu 1995, Raynor et al. 1994). It is possibly mediated by a post-receptor mechanism, such as phosphorylation (King and Cuatrecasas 1983, Kandel et al. 1995). Down-regulation, on the other hand, is a decrease in the number of membrane receptors due to internalization (Niedel 1983, Bhargava et al. 1991). The reduced sensitivity to agonist-induced responses under physiological or pharmacological circumstances can be a result of receptor desensitization and/or receptor down-regulation (Bhargava et al. 1991, Polastron et al. 1994).

During hibernation, μ receptor binding decreased significantly in the medial septum (Chapter Four). This decline in μ receptor binding is probably the result of an increase in β-endorphin content in the septum, as demonstrated by tissue homogenate (Chapter Five). Although the μ receptor binding is unaltered in the lateral septum, *in vivo* microdialysis revealed an elevation in extracellular β-endorphin concentration as well (Chapter Five). However, extracellular β-endorphin concentration is even higher in the inter-bout euthermia than during hibernation, which suggests that the increase in β-endorphin concentration may have been only initiated during hibernation and does not peak until the completion of arousal. Thus a short term exposure of μ receptor to relatively low β-endorphin activity could not induce the down-regulation of μ receptors in the lateral septum during hibernation. In the hippocampal formation, a decrease in μ receptor binding is only observed in the CA3 region whereas the receptor density in the CA1 and dentate gyrus is about the same in hibernating squirrels as that in non-hibernating animals (Chapter Four). This supports the observation that the inhibitory effect of the μ agonist, DAGO, on 5-HT release from the hippocampal slices remains during hibernation (Chapter Two) since 5-HT terminals are densely located in the dentate gyrus but only moderately in CA1 and CA3 of the hippocampal formation (Moore and Halaria 1975, Parent et al. 1981). The reduction of μ agonist inhibition on 5-HT release from the CA3 region may be masked by an unaltered response in the dentate gyrus which is compactly innervated by 5-HT terminals. In addition, an increase in β-endorphin immunoreactivity was also observed during hibernation. However, this increase in μ opioid activity was not reflected by the μ receptor binding (Chapter Four) in the preoptic area because it is only a small portion of the hypothalamus and may have not changed correspondingly with the other nuclei. In contrast to the septum, a decrease in β-endorphin content is shown in the medulla of hibernating Columbian ground squirrels (Chapter Five). This observation is in accordance with the observed decrease of β-endorphin in the midbrain and pons of hibernating golden-mantled ground squirrels (Mueller et al. 1990).

Similar to β-endorphin, an elevation of met-enkephalin content in the septum of hibernating animals is also evident. This supports previous immunochemical studies showing an increase in met-enkephalin-immunoreactivity in the perikarya of the lateral septum (Nurnberger et al. 1991). It is also in agreement with the observed decrease in δ receptor binding (Chapter Four), reduced sensitivity in thermoregulatory effects to external met-enkephalinamide administration (Lee et al. 1989), and a decreased efficacy of met-enkephalinamide on stimulating *in vivo* 5-HT release as measured by microdialysis (Chapter Two). Although the leu-enkephalin content decreased slightly in the septum, because of its low concentration compared to met-enkephalin, its contribution to the overall alteration in endogenous δ agonists is insignificant. In the hippocampus, on the other hand, a decrease in δ receptor binding in the CA3 and hippocampal fissure of the hippocampal formation echoes the attenuation of inhibitory modulation of 5-HT release in the hibernating animals. However, only a slightly increased met-enkephalin content in the overall hippocampal region was observed, probably because of the masking by the insignificant change in the CA1 and dentate gyrus.

Unlike β-endorphin and the enkephalins, a very high claustral content of dynorphin A-like immunoreactivity was observed in the non-hibernating ground squirrels (Chapter Five). During hibernation, the dynorphin A immunoreactivity was even higher (Chapter Five), probably accounted for the observed decrease of claustral κ receptor binding (Chapter Four). Similarly, an increased hippocampal dynorphin A-immunoreactivity during hibernation (Chapter Five) is in accordance with the observed attenuation of κ agonist stimulation of 5-HT release from the hippocampal slices during hibernation. Autoradiographic study further confirms the decrease in κ receptor density in the CA3 region in the hippocampus (Chapter Four). Further, a rise in extracellular concentration of dynorphin A-like peptide in the lateral septum, indicated by *in vivo* microdialysis, is also in agreement with a decline in κ receptors in the same region. However, the absence of an expected significant increase in dynorphin A-immunoreactivity in the lateral septum is probably masked by the lack of change in the medial septum, when both areas were homogenized together. In contrast to the change in septal β-endorphin concentration, septal dynorphin A concentration is the highest in the hibernating state, intermediate in the inter-bout euthermia, and lowest in the non-hibernating phase.

Peripherally, the synthesis and secretion of opioids have been demonstrated from several sources, such as the peripheral nervous system and numerous organs or organ systems (Akil et al. 1984, 1988, Hokefelt et al. 1984, Alessio et al. 1989, Negri et al. 1989). Locally, opioids may only elicit their physiological functions by activating receptors in the vicinity whereas circulating opioids can interact systemically with remote receptors or even with receptors in the CNS via specific transporters for crossing the brain-blood-barrier (Schick and Schusdziama 1985, Banks and Kastin 1990, Begley 1994). Therefore, circulating endogenous opioids may have important multi-functional roles in regulating hibernation.

During hibernation, the plasma concentrations of met-enkephalin and immunoreactive dynorphin A were elevated whereas that of leu-enkephalin was unaltered (Chapter Five). In contrast, the plasma concentration of β-endorphin-like peptide of the hibernating ground squirrels decreased. This is not unexpected since previous studies have shown that the β-endorphin content in the pituitary, the main source of plasma β-endorphin, decreases significantly during hibernation (Mueller et al. 1990). Similar to central endogenous opioids, enkephalin concentrations are also about two orders of magnitude higher than β-endorphin and dynorphin A-like peptides in the plasma.

Taken together, the selective increases in endogenous opioids in certain limbic nuclei during hibernation correlate well with the observed decrease in efficacy of corresponding receptors. These results confirm previous observations of reduction in dihydromorphine, a μ ligand with low selectivity, binding in the septum and hippocampus (Beckman et al. 1986) and decrease in binding of a κ ligand, EKC, in the brain homogenate of hibernating animals (Aloia et al. 1985). The present results also provide an explanation to the reported absence of-state-dependent changes of opioid receptor binding (Wilkinson et al. 1986, Koleava et al. 1989) since they were based on whole brain or large sized tissue homogenates rather than small regions or nuclei.

Our observed decrease in opioid receptor binding does not seem to be a result of general CNS depression during hibernation because pentobarbitone-induced general depression does not prevent the development of physical dependence of animals to morphine (Beckman et al. 1993a), whereas physical dependence to morphine can not be induced in the hibernating state (Beckman et al. 1981). It is, therefore, suggested that the lack of physical dependence to exogenous morphine during hibernation is the result of decreased opioid receptor efficacy induced by increased endogenous opioid activity. In addition, the increase in endogenous opioids during hibernation does not seem to be induced by cold because cold stimulus decreases hypothalamic dynorphin level (Morely et al. 1982), which is not seen during hibernation. Physical stress-induced changes in met-enkephalin and dynorphin (Kurumaji et al. 1987, Nabeshima et al. 1992) do not follow the same pattern as seen in hibernation, either.

The state-dependent increase in opioid contents in tissue homogenates is further substantiated to be of functional significance since they correlate with their extracellular concentration and apparent turnover as demonstrated by *in vivo* microdialysis (Chapter Five). These observations tend to indicate that the-state-dependent changes in endogenous opioid activity may be regulated at the gene expression level by a circannual rhythmic mechanism in the limbic system. The observed specific changes in endogenous opioid activities may imply their functional importance in regulating different physiological processes in hibernation.

Possible functional roles of regulatory changes in endogenous opioids and opioid receptors in the hibernation cycle

An elevation of endogenous opioids can be a result of increased production/gene expression and/or reduced degradation. Gene expression of three opioid families has been demonstrated to be regulated by neural activity and neurochemical stimulations (Uhl et al. 1988, Pelletier 1993, McMurray et al. 1992). Enhanced neuronal activity in the limbic system prior to and/or during hibernation is speculated to be able to modulate opioid gene expression. However, gene expression of one opioid family is differentially regulated from the others to accomplish the desired specific physiological functions (Comb et al. 1992, L'Hereault and Barden 1991, McMurray et al. 1992), such as food intake, thermoregulation, cardiovascular responses, and modulating release of other neurotransmitters/ hormones (for review see Chapter One). The expression for each of them may be regulated by various internal or external cues affecting different nuclei of the limbic system.

A. Prodynorphin family and κ receptors

Expression of prodynorphin has been demonstrated to be regulated by relevant physiological or neural stimuli, such as inflammation, seizure, and administration of excitatory neurotransmitters (Ladarola et al. 1988, Naranjo et al. 1991) which in turn leads to alterations of the dynorphin level in the corresponding nuclei (Gall 1988). Based on the results of the present findings, it is postulated that on the transition from the non-hibernating to the hibernating phase, prodynorphin gene expression is enhanced by an endogenous CNS mechanism. This is supported by our observed extracellular dynorphin A immunoreactivity in the septum being the highest during hibernation, intermediate in the inter-bout euthermia, and lowest in the non-hibernation phase. Physiologically, the onset of the hibernation season is characterized by hyperphagia and deposition of body fat which causes a rapid gain in body weight followed by hypophagia and weight plateau. Activation of hypothalamic and extra-hypothalamic κ receptors stimulates food and fat intake and increases body weight (Nizielski et al. 1986, Romsos et al. 1987, Stanley et al. 1989, Bhargava and Matwyshyn 1993), whereas high dose of κ agonist reduces feeding (Yim and Lowy 1984, Nizielski et al. 1986). The postulated increase in concentration of dynorphin A-like κ peptides at the onset of hibernation season, may result in stimulation of food intake leading to the fall fattening. However, a further increase in dynorphin A level induces hypoghagia and body weight plateau or even a decrease prior to hibernation. This is in accordance with previous studies that cortical, hypothalamic, and striatal dynorphin content increased during the fall fattening season and reached its highest concentration at the onset of anoxia (Nizielski et al. 1986). The claustrum, as part of the limbic system, has been shown to modulate sensory motor and somatosensory inputs and is speculated to be involved in appetitive behavior responses (Simpson and Fitch 1988). A higher dynorphin A immunoreactivity in this area at the early phase of the hibernation season may be related to its proposed role in food intake. Furthermore, central and peripheral κ opioids have been shown to induce hypothermia, sedation, bradycardia, and respiratory depression (Snyer 1986, Stanley et al. 1989, Adler et al. 1988, 1991, 1992, Burks 1991, Wollemann et al. 1993, Olson et al. 1994). An increase in dynorphin A-like immunoreactivity in both CNS and plasma may induce the overall autonomic depression in the entry phase of the hibernation season.

As shown in Chapter Two, the κ receptor may also act with 5-HT in the possible regulation of hibernation. 5-HT is one of the neurotransmitters which is considered to be important in the entry into and maintenance of hibernation.

Evidence supporting such a role includes an increase in 5-HT turnover during hibernation or winter torpor (Novotona et al. 1975, Lin and Pivorun 1989), the prevention of onset of hibernation by chemical or electrical lesioning of specific 5-HT-producing neurons in the median raphe nucleus (Spafford and Pengelley 1971, Canguilhem et al. 1986), and an increase in 5-HT synthesizing enzyme prior to hibernation (Popova et al. 1993). The increased endogenous κ opioid, dynorphin A, at the onset of hibernation season stimulates 5-HT release. Increased 5-HT activity suppresses eating (Leibowitz 1986, Rauru et al. 1993), enhances inhibitory neural activity in the septo-hippocampal complex (Milner and Veznedaroglu 1993), and further facilitates the inhibition of the hypothalamus and the brain stem reticular formation for transition to hibernation. As 5-HT terminals directly modulate septo-hippocampal neuronal activity (Milner and Veznedaroglu 1993), increased inhibitory activity may facilitate the κ induced autonomic depression, resulting in entry into hibernation.

It has been shown that neuronal activity or electrical activity in the perforant pathway, a major input to the hippocampus from entorhinal cortex, may suppress prodynorphin expression in the dentate gyrus of hippocampal formation (Comb et al. 1992). Further, increase in κ activity itself also reduces the release of dynorphin to the synaptic gap by autoregulation (Nikolarakis et al. 1989). It is reasonable to hypothesize that a drastic raise in dynorphin A level prior to hibernation will in turn inhibit further prodynorphin expression (Romualdi et al. 1991) and release. The increase in dynorphin A immunoreactivity in the entry phase of hibernation also down-regulates κ receptors during hibernation, which accounts for the reduced sensitivity and/or indifference to κ ligands in deep hibernation or arousing phase. In concert with the reduction of gene expression, the gradual decrease in κ activity occurs during a hibernation bout. The slowly declined extracellular dynorphin concentration together with the reduced κ receptor efficacy causes a gradual reduction in inhibitory activity of the septo-hippocampal complex to a point that it can on longer maintain the animal in hibernation and the animal arouses. This hypothesis is supported by observations of plasma dynorphin A immunoreactivity variability during a hibernation bout (unpublished data). Preliminary studies with continuous blood sampling, using jugular cannulation, throughout a hibernation bout indicate that the highest dynorphin A immunoreactivity was reached on the first day of a hibernation bout and gradually declined as the hibernation bout progresses. Dynorphin A content in plasma reached the lowest level during arousal. Therefore, it appears that both central and peripheral dynorphin A activities increase during inter-bout euthermia. Onset of another hibernation bout results when dynorphin reaches its highest level.

B. Proenkephalin family and δ receptors

Proenkephalin gene expression has been demonstrated to be stimulated by electrical or chemical stimuli, neural activity, and mediators of the second messenger systems (Comb et al. 1992). Increased neural activity in the limbic system prior to hibernation or during the entry phase of hibernation may be a stimulatory factor for proenkephalin gene expression, resulting in a gradual increase in enkephalin levels in the CNS. The observed increase in met-enkephalin content in the septal region (Chapter Five) may have some functional roles in regulating hibernation cycle. It may enhance the release of 5-HT (Chapter Two), resulting in lowering of Tb (Chapter Three). In view of the inhibitory nature of met-enkephalin, further increase in met-enkephalin activity in the septal area may interact with 5-HT to impose an inhibitory signal from the septo-hippocampal complex to the reticular formation. It is, thus, possible that endogenous enkephalinergic system may elicit one or both above mentioned effects to facilitate the entry into and/or maintenance of hibernation.

Thyrotropin-releasing-hormone (TRH), on the other hand, is presumed to be an arousal factor. It has been shown previously that hippocampal injection of TRH causes premature arousal from hibernation (Stanton et al. 1980, 1981). Further, inclusion of TRH to septal slices of hibernating ground squirrels induces a profound increase in the frequency of spontaneous activity of medial septal neurons (Belousov and Belousova 1992). It has been demonstrated that TRH antagonizes pentobarbital narcosis and induces arousal through activating the septo-hippocampal system (Kalivas et al. 1981). These observations raise the possibility that arousal from hibernation can be initiated by an increase in excitatory activity of TRH in the septo-hippocampal complex to remove the prevailing inhibitory suppression on BSRF and hypothalamus (Belousov and Belousova 1992, Belousov 1993). This notion is supported by the recent finding of seasonal and state-dependent changes in TRH receptor binding (Stanton et al. 1992), showing a seasonal decrease in TRH receptor binding in some hypothalamic nuclei comparing winter euthermic with summer euthermic animals and a state-dependent decrease in hypothalamic TRH binding and an increase in amygdala TRH binding when hibernating animals were compared with winter euthermic animals. Enkephalin- and TRH-containing neurons have been demonstrated to co-exist in the hypothalamic region (Ceccatelli et al. 1989, Tang and Mah 1991) and some project to the lateral septum (Ishikawa et al. 1986, Merchenthaler 1991). Furthermore, enkephalins have been shown to have an inhibitory effect on central TRH activity (Jordan et al. 1986, Krumins 1988, Mitsuma et al. 1993). These observations may lead to a suggestion that the increase in enkephalin activity may facilitate and maintain hibernation by inhibiting endogenous TRH activity. This may possibly be the case since a decrease in TRH concentration in most of the limbic region is observed in the hibernating ground squirrels (Stanton et al. 1982).

Increased enkephalin activity may down-regulate δ receptors (Prather et al. 1994) during hibernation. With the prolongation of the hibernation bout, the increased δ activity might also reduce the expression of proenkephalin gene via negative feedback (Uhl et al. 1988) and the release of enkephalins by activating presynaptic autoreceptors (Nikolarakis et al. 1989), Consequently, the inhibitory effect of enkephalins on TRH may diminish with time in hibernation and arousal ensues. Therefore, an increase in δ opioid activity in the early phase of hibernation facilitates hibernation by enhancing 5-HT and/or suppressing TRH activities whereas a decrease in δ opioid activity in late hibernation bout may lead to spontaneous arousal by removing its inhibitory effect on TRH activity. This speculation that enkephalins may facilitate the entry into and maintenance of hibernation is further supported by previous observations that premature arousal initiated when δ opioid activity was blocked by the non-specific opioid antagonists naloxone (Margules 1979, Beckman and Liados-Eckman 1985) and naltrexone (Kromer 1980) or the δ antagonist naltrindole (Yu and Cai 1993).

C. POMC family and μ receptors

Most of the POMC gene expression has been shown to be located in the arcuate nucleus of hypothalamus, the main source of central β-endorphin, and the anterior pituitary, which releases β-endorphin to blood (Genazzani et al. 1984, Negri et al. 1989). The expression is regulated by dopamine and other neurotransmitters/ hormones, including opioids (L'Hereault and Barden 1991, Pelletier 1993, Yebenes and Pelletier 1993). However, the expression is differentially regulated in the arcuate nucleus and pituitary (Pelletier 1993).

Since β-endorphin binds to μ, δ, and a proposed ε receptors, its physiological effects are the result of activation of all bound receptors and are more complicated. However, as β-endorphin concentration is usually two orders of magnitude lower than that of enkephalins in the same region in both non-hibernating and hibernating ground squirrels (Chapter Five) and rats (Cuello 1983), the physiological responses induced by β-endorphin, if not seen by δ activation, are mostly likely non-δ and supposedly via μ receptors. In the hibernating ground squirrels, septal β-endorphin content is intermediate between that of euthermic non-hibernating and euthermic inter-hibernation bout animals

(Chapter Five). Further, unlike κ and δ receptors (Chapter Two), μ receptors are not down-regulated during hibernation, as evidenced by its unaltered binding (Chapter Four) and its persistent inhibitory effect on 5-HT release, suggesting a low μ activity during hibernation. The gradual increase in β-endorphin level from hibernating state to inter-bout euthermic state, in the septal region, may imply its role in arousal. It has been demonstrated that stimulation of central μ receptors elevates epinephrine and norepinephrine concentration in blood and enhances sympathetic drive (Van Loon et al. 1989). Increase of sympathetic activity has been shown to be a critical component of the arousal process (Lyman 1982). Moreover, activation of μ receptors in the hypothalamic paraventricular nuclei has been demonstrated to increase blood pressure and tachycardia (Bachelard and Pitre 1995). Therefore, the observed increase in β-endorphin immunoreactivity in the hypothalamus (Chapter Five) during hibernation may have a role in inducing arousal. In addition, slowly increased β-endorphin concentrations with the progression of hibernation bout (Chapter Five) could suppress 5-HT activity (Chapter Two, Passarelli and Costa 1988, Yoshioka et al. 1993). The attenuation of 5-HT activity may work together with elevated TRH activity, caused by reduction of δ activity, to induce arousal.

Interestingly, μ receptors have been shown to elicit opposite effects to κ receptors in many physiological and pharmacological situations, such as in thermoregulation (Spencer et al. 1990), modulating 5-HT (Cui et al. 1993) and dopamine release (Di Chiara and Imperato 1988), firing-rate of dopamine cells (Walker et al. 1987), modulating locomotor activity (Mansour et al. 1988), and fluid regulation (Mansour et al. 1988). *Mu* agonist-induced increase in dopamine metabolism (Kim et al. 1987) and impairment of spontaneous alteration performance (Itoh et al. 1994) have been shown to be antagonized by κ agonists. In addition, down-regulation of κ receptor binding, induced by chronic administration of κ agonists, causes a simultaneous up-regulation of μ binding (Morris and Herz 1989). The difference in μ and κ receptor-elicited physiological effects may be a result of coupling to different ion channels. It has been shown that μ receptors couple to potassium channels (North et al. 1987), whereas κ receptors couple to calcium channels (Wollemann 1990. Childers 1991). It is, thus, quite possible that μ receptors play a different role from κ receptors in regulating hibernation. The speculation that activation of μ receptors mediates arousal from hibernation is supported by the recent observation that icv infusion of β-endorphin in hibernating ground squirrels induces premature arousal (Beckman et al. 1993b).

Being very complicated systems, endogenous opioids and receptors also interact and cross-react with each other and with other neurotransmitters and hormones to modulate gene expression (Reimer and Holt 1991, Romualdi et al. 1991, Lason et al. 1992), the release (Ueda et al. 1987, Nikolarakis et al. 1989, Kelly et al. 1990) of the three families of opioid peptides, and the regulation of opioid receptor sensitivity and /or composition (Simantov et al. 1989, Bhargava et al. 1991, Polastron et al. 1994, Prather et al. 1994, Raynor et al. 1994). For instance, chronic administration of a κ agonist, U50488, induces a decrease of plasma β-endorphin content (Milanes et al. 1991), which may suggest that the decrease in plasma β-endorphin concentration during hibernation is a result of increased κ opioid dynorphin activity. Moreover, opioid systems are not in isolation from other neurotransmitters. They have been demonstrated to modulate the activity of other neurotransmitters and hormones (Illes 1989) and be modulated by other neurochemicals (McMurray et al. 1992, Pelletier 1993) as well. It has been shown that increase in 5-HT activity by administration of 5-HT precursor or uptake inhibitor elevated hypothalamic β-endorphin immunoreactivity (Majeed et al. 1985). The possibility that our observed increase in β-endorphin content in the hypothalamus during hibernation is a consequence of increased 5-HT activity stimulated by κ and/or δ opioids cannot be ruled out. Norepinephrine, acetylcholine, adenosine, vasopressin, and melatonin have also been indicated to be possibly relate to the regulation of hibernation (Stanton et al. 1984, 1987, Wang 1989, Hermes et al. 1993, Lee et al. 1993). Therefore, it is possible that opioids may regulate hibernation through activating or inhibiting other neurotransmitters and hormones as well.

In conclusion, the state-dependent increase of various opioid activities indicates their functional roles in regulating hibernation. Increased κ opioid activity at the onset of the hibernation season stimulates food intake and body weight gain. Further enhancement of κ activity stimulates 5-HT activity, which, in concert with central and peripheral κ opioids, leads to the suppression of BSRF from the septo-hippocampal complex and general depression of autonomic functions. Furthermore, increase in δ activity, prior to or in the early stage of a hibernation bout, facilitates the transition to and maintenance of hibernation, by enhancing 5-HT activity and suppressing TRH excitatory activity in the septo-hippocampal complex. As the hibernation bout progresses, the κ and δ inhibitory effects diminish because of negative feedback and receptor down-regulation until the inhibitory activity is overcome by excitatory TRH impact. In addition, with the facilitation of β-endorphin-induced increase in sympathetic drive, arousal initiates. However, the regulation of hibernation is probably much more complex an event than we hypothesized here. Opioids must have acted in concert with other neurochemicals to regulate the complicated physiological changes throughout a hibernation bout as well as the seasonal hibernation cycle. The proposed hypothesis has to be verified by further investigations into opioid peptide and receptor gene expression throughout the hibernation cycle, and by altering hibernation patterns through the administration of various opioid agonists and antagonists during the various time periods of a hibernation cycle. Only after such systematic investigations plus the interaction of opioids with other neurochemicals, can we begin to understand the key aspects regulating the hibernation precess.

References

Adler M. W., Geller E. B., Rosow C. E., and Cochin J. (1988) The opioid system and temperature regulation. Ann Rev Pharmacol Toxicol 28:429-49

Adler M.W., Bradley E., Martinez R., and Geller E.B. (1991) Production of hypothermia in the guinea pig by a kappa-agonist opioid alone and in combination with chlorpromazine. Pharmacol Biochem Behav 40:129-132

Adler M.W. and Geller E.B. (1992) Physiological functions of opioids: temperature regualtion. Handbook of Experimental Pharmacology. Vol. 104/II Opioids II Herz A., Akil H., and Simon E.J. (eds), pp 205-238, Pergamon Press, New Yrok.

Akil H., Watson S. T., Yang E., Lewis M. E., Khaehaturian H. and Waller J. M. (1984) Endogenous opioids: biology and function. Ann Rev Neurosci 7:223-255

Akil H., Bronstein D., and Mansour A. (1988) Overview of the endogenous opioid systems: anatomical, biochemical and functional issues. Endorphins, Opiates and Behavioral Processes Rodgers R.J. and Cooper S.J. (eds), pp 1-23, Chichester, New York.

Alessio L., Salar F., Battaini F., Govoni S., and Trabucchi M. (1989) Endogenous opioids in human biological fluids: in search of a functional role. Opioid Peptides in Biological Fluids Genazzani A.R. and Negri M. (eds), pp 45-52, Parthenon, Lancs.

Aloia R.C., Vasquez B.J., and Tucker S.L. (1985) Opioid receptors in the brain of active and hibernating ground squirrels, *Spermophilus lateralis.* Abstracts for Living in the Cold. (Fallen Leaf Lake, California) Heller H.G., Musacchia X.J., and Wang L.C.H. (eds), p 211

Bachelard H. and Pitre M. (1995) Regional haemodynamic effects of μ-, δ-, and κ-opioid agonists microinjected into the hypothalamic paraventricular nuclei of conscious, unrestrained rats. Br J Pharmacol 115:613-621

Banks W.A. and Kastin A. (1990) Peptide transport systems for opiates across the blood-brain barrier. Am J Physiol 259 (Endocrinol Metab 22):E1-E10

Beckman A. L., Llados-Eckman C., Stanton T. L., and Adler M. W. (1981) Physical dependence on morphine fails to develop during the hibernating state. Science 212:527-529

Beckman A.L. and Stanton T.L. (1982) Properties of the CNS during the state of hibernation. The neural basis of behaviour Beckman A.L. (ed), 19-45, Spectrum, New York.

Beckman A. L., and Llados-Eckman C. (1985) Antagonism of brain opioid peptide action reduces hibernation bout duration. Brain Res 328:201-205

Beckman A., Dean R. R.,and Wamsley J. K. (1986) Hippocampal and cortical opioid receptor binding: changes related to the hibernation state. Brain Res 386:223-231

Beckman A.L., Beaver T.A., and Lewis F.A. (1993a) Morphine physical dependence in the hibernator: cental nervous system mechanisms underlying the development of dependence remain functional during depression induced by pentobarbital anaesthesia. Life Sci. 52:1079-1086

Beckman A., Kim M.M., and Newman J.R. (1993b) β-Endorphin action during hibernation: dose-dependent reduction of bout length produced by intracerebroventricular infusion in ground squirrels (*Citellus lateralis*). Living In the Cold III. p 4

Belousov A.B. and Belousova J.V. (1992) State-dependent and state-independent excitibility of the medial septal neurons in brain slices of ground squirrel, *Citellus undulatus.* Neuroscience 38:599-608

Belousov A.B. (1993) On possible mechanisms of the septohippocampal system participation in the neural control of hibernation. Life in the Cold: Ecological, Physiological, and Molecular Mechanisms. Carey C., Florant G.L., Wunder B.L., and Horwitz B. (eds), pp 291-296, Westview Press, Colorado.

Bhargava H.W. and Das S. (1986) Evidence for opioid action at the brain receptors for thyrotropin-releasing hormone. Brain Res 368:262-267

Bhargava H.N., Gulati A., and Ramarao P. (1991) Down-regulation of brain and spinal cord κ-opiate receptors in spontaneously hypertensive, Wistar-Kyoto normotensive, and Sprague-Dawley rats by chronic treatment with U-50,488H. Biochem Pharmacol 42:25-29

Bhargava H.N. and Matwyshyn G.A. (1993) Effect of N^G-monomethyl-L-arginine on tolerance to U-50,488H in the rat. Neurosci Abstr 19:1461

Boulant J.A., Curras M.C., and Dean J.B. (1989) Neurophysiological Aspects of thermoregulation. Advances in Comparative and Environmental Physiology Vol.4 Wang L.C.H. (ed), pp 117-160, Springer-Verlag, Berlin.

Brush F.R. and Shain C.N. (1989) Endogenous opioids and Behavior. Psychoendocrinology Brush F.R. and Levine S. (eds), pp 379-435, Academic Press, San Diago.

Burks T.F. (1991) Opioids and opioid receptors in thermoregulation. Thermoregulation: Pathology, Pharmacology and Therapy Schonbaum E. and Lomax P. (eds),pp 489-508, Pergamon Press, New York.

Canguilhem B., Miro J.L., Kemph E., and Schmitt P. (1986) Does serotonin play a role in entrance into hibernation? Am J Physiol 22:701-708

Ceccatelli S., Eriksson M., and Hokfelt T. (1989) Distribution and coexistence of corticotropin-releasing factor-, neurotensin-, enkephalin-, cholecystokinin-, galanin-, and vasoactive intestinal polypeptide/peptide histidine isoleucine-like peptides in the parvocellular part of the paraventricular nucleus. Neuroendocrinology 49:309-323

Childers S.R. (1991) Opioid receptor-coupled second messenger systems. Life Sci 48:1991-2003

Cohen D.H. and Sherman S.M. (1983) The autonomic nervous system and its central control Physiology Berne R.M. and Levy M.N. (eds), pp 314-335, C V Mosby, St. Louis.

Comb M.J., Kobierski L., Chu H.M., and Tan Y. (1992) Regulation of opioid gene expression: a model to understand neural plasticity. Molecular Approcahes to Drug Abuse Research Volume II: Structure, Function, and Expression. NIDA Research Monograph Vol 126 Lee T.N.H. (ed), pp 98-112, NIDA publication, Rockville.

Cuello A.C. (1983) Central distribution of opioid peptides. Med Bull 39:11-16

Cui Y., Lee T.F., Kramarova L.I., and Wang L.C.H. (1993) The modulatory effects of *mu* and *kappa* opioid agonists on 5-HT release from hippocampal and hypothalamic slices of euthermic and hibernating ground squirrels. Life Sci 53:1957-1965

DePaoli A.M., Hurley K.M., Yasada K., Reisine T., and Bell G. (1994) Distribution of κ opioid receptor mRNA in adult mouse brain: an *in situ* hybridization histochemistry study. Mol Cell Neurosci 5:327-335

Di Chiara G. and Imperato A. (1988) Opposite effects of *mu* and *kappa* opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. J Pharmacol Exp Ther 244:1067-1080

Gall C. (1988) Seizures induce dramatic and distinctly different changes in enkephalin, dynorphin, and CCK immunoreactivities in mouse hippocampal mossy fibers. J Neurosci 8:1852-1862

Genazzani A.R., Petraglia F., Nappi G., Martignoni E., Leo M.D., and Facchinetti F. (1984) Endorphins in peripheral plasma: origin and influencing factors. Central and Peripheral Endorphins: Basic and Clinical Aspects Muller E.E. and Genazzani A.R. (eds), pp 89-98, Raven Press, New York.

George S.R., Zastawny R.L., Brones-Urbina R., Cheng R., Nguyen T., Heiber M., Kouvelas A., Chan A.S., and O"Dowd B.F. (1994) Distinct distribution of mu, delta, and kappa opioid receptor mRNA in rat brain. Biochem Biophys Res Commun 205:1438-1444

Gouarderes C., Tellez S., Tafani J.A., and Zajac J.M (1993) Quantative autoradiographic mapping of delta-opioid receptors in the rat central nervous system using $[^{125}I][D.Ala^2]$deltorphin-I. Synapse 13:231-240

Heller H. C. (1979) Hibernation: neural aspects. Ann Rev Physiol 41:305-321

Herkenham M. and McLean S. (1988) The anatomical relationship of opioid peptides and opiate receptors in the hippocampi of four rodent species. Opioids in the Hippocampus McGinty J.F. and Friedman D.P. (eds), pp 33-47, NIDA publication, Rockville.

Hermes M.L.H.J., kalsbeek A., Kirsch R., Buijs R.M., and Pevet P. (1993) Induction of arousal in hibernating European hamsters (*Cricetus crietus* L.) by vasopressin infusion in the lateral septum. Brain Res 631:313-316

Hokfelt T., Vincent S.R., Dalsgaard C.J., Herrera-Marschitz M., Ungerstedt U., Schultzberg M., Chiristensson I., and Terenius L. (1984) Some aspects on distribution and role of opioid peptides in the central and peripheral nervous system. Central and Peripheral Endorphins: Basic and Clinical Aspects Muller E.E. and Genazzani A.R. (eds), pp 1-16, Raven Press, New York.

Illes P. (1989) Modulation of transmitter and hormone release by multiple neuronal opioid receptors. Rev Physiol Biochem Pharmacol 112:141-233

Ishikawa K., Taniguchi Y., Kurosumi K., and Suzuki M. (1986) Origin of septal thyrotropin-releasing hormone in the rat. Neuroendocrinology 44:54-58

Itoh J., Ukai M., and Kameyama T. (1994) Dynorphin A-(1-13)) potently improves the impairment of spontaneous alteration performance induced by the μ-selective opioid receptor agonist DAMGO in mice. J Pharmacol Exp Ther 269:15-21

Johnston D. and Wu S.M.S. (1995) Foundations of Cellular Neurophysiology pp 373-374, MIT Press, Cambridge.

Jordan D., Veisseire M., Borson-Chazot F. and Mornex R. (1986) In vitro effects of endogenous opiate peptides on thyrotropin function: inhibition of thyrotropin-releasing hormone release and absence of effect on thyrotropin release. Neurosci Lett 67:289-294

Kalivas P.W., Simasko S.M., and Horita A. (1981) Effect of septohippocampal lesions on thyrotropin-releasing hormone antagonism of pentobarbtial narcosis. Brain Res 222:253-265

Kandel E.R., Schwartz J.H., and Jessell T.M. (1995) Essentials of Neural Science and Behavior pp 260-261, Appketon & Lange, Norwalk.

Kelly M.J., Loose M.D., and Ronnekleiv O.K. (1990) Opioids hyperpolarize β-endorphin neurons via μ-receptor activation of a potassium conductance. Neuroendocrinology 52:268-275

Khachaturian H., Lewis M.E., Schafer M.K.-H., and Watson S.J. (1985) Anatomy of the CNS opioid system. TINS 8:111-119

Kilduff T. S., Radlke C. D., and Heller H. C. (1986) Neural activity during mammalian hibernation. Living in the Cold Heller H.C., Musacchia X.J., and Wang L.C.H. (eds), pp 215-223, Elsevier, Amsterdam.

Kilduff T. S., and Heller H. C. (1989) Neurochemical studies of hibernation. Living in the Cold II Malan A. and Canguilhem B. (eds), pp 467-475, John Libbey Eurotext, Montrouge.

Kim H.S., Iyengar S., and Wood P.L. (1987) Reversal of the actions of morphine on mesocortical dopamine metabolism in the rat by the kappa agonist MR-2034: tentative mu-2 opioid control of mesocortical dopaminergic projections. Life Sci 41:1711-1715

King A.C. and Cuatrecasas P. (1983) Adsorptive pinocytosis of epidermal growth factor: studies of its relevance to mitogensis. Receptor-Mediated Endocytosis. Receptors and Recognition Vol.15, Cuatrecasas P. and Roth T. (eds), pp 47-81, Chapman and Hall, London.

King T.R. and Nance D.M. (1986) Differential effects of neurotoxic lesions on psychoneuroendocrine functions. Pharmacol Biochem Behav 24:107-114

Kolaeva S.G., Kramarova L.I., and Yarying K.N. (1989) The role of opioids and TRH in the hibernation bout/arousal cycle of ground squirrels *Citellus undulatus*. Living in the cold II Malan A. and Canguilhem B. (eds), pp 461-466, John Libbey Eurotext, Montrouge.

Kramarova L.I., Kolaeva S.G., Yukhanaov R.Y., and Rozhanets V.V. (1983) Content of DSIP, enkephalins, and ACTH in some tissue of active and hibernating ground squirrels. Comp Biochem Physiol 74C:31-33

Kromer W. (1980) Naltrexone influence on hibernation. Experientia 36:581-582

Krumins S.A. (1988) Simultaneous modulation of TRH and opioid receptor binding in rat spinal cord after chronic naloxone administration. Neuropeptides 11:47-52

Kurumaji A., Takashima M., and Shibuya H. (1987) Cold and immobilization stress-induced changes in pain responsiveness and brain met-enkephalin-like immunoreactivity in the rat. Peptides 8:355-359

Ladarola M.J., Douglass J., Civelli O., and Naranjo J.R. (1988) Differential activation of spinal cord dynorphin and enkaphalin neurons during hyperalgesia: evidence using cDNA hybridization. Brain Res 455:205-212

Lason W., Przewlocka B., and Przewlocki R. (1992) The effects of excitatory amino acids on proenkephalin and prodynorphin mRNA levels in the hippocmapal dentate gyrus of the rat: an in situ hybridization study. Mol Brain Res 12:243-247

Lee M.G., Chrobak J.J, Sik A., Wiley R.G., and Buzsaki G. (1994) Hippocampal theta activity following selective lesion of the septal cholinergic system. Neuroscience 62:1033-1047

Lee T. F., Nurnberger F., Jourdan M. L. and Wang L. C. H. (1989) Possible involvement of septum in seasonal changes in thermoregulatory responses to met-enkephalinamide in ground squirrels. Thermoregulation: Research and clinical applications Schonbaum E. and Lomax P. (eds), pp 200-203, Kruger, Basel.

Lee T.F., Nurnberger F., and Wang L.C.H. (1993) Possible involvement of endogenous adenosine in hibernation. Life in the Cold: Ecological, Physiological, and Molecular Mechanisms Carey C., Florant G.L., Wunder

B.A., and Horwitz B. (eds), pp 315-322, Westview Press, Colorado.

Leibowitz S.H. (1986) Brain monoamines and peptides: role in the control of eating behavior. Federation Proc 45:1396-1403

L'Hereault S. and Bardent N. (1991) Regulation of proopiomelaocortin messenger RNA concentrations by opioid peptides in primary cell cultures of rat hypothalamus. Mol Brain Res 10:115-121

Lin LH. and Pivorun E.B. (1989) Analysis of serotonin, dopamine and their metabolites in the caudate putamen, the suprachiasmatic nucleus and median raphe nucleus of euthermic and torpid deermice, *Peromyscus maniculatus*. Pharmacol Biochem Behav 33:309-314

Lyman C.P., Wills J.S., Malan A., and Wang L.C.H. (eds) (1982) Hibernation and Torpor in Mammals and Birds Academic Press, New York.

Lynch G.S., Rose G., and Gall C.M. (1977) Anatomical and functional aspects of the septo-hippocampal projections. Functions of the Septo-Hippocampal System. Ciba Foundation Symposium 58, pp 5-19, Elsevier, Amsterdam.

Majeed N.H., Lason W., Przewlocka B., and Przewlocki R. (1985) Differential modulation of the beta-endorphin and dynorphin systems by serotonergic stimulation in the rat. Neuropeptides 5:563-566

Mansour A., Lewis M.E., Khachaturian H., Akil H., and Watson S.J. (1986) Pharmacological and anatomical evidence of selestive mu, delta, and kappa opioid receptor binding in rat brain. Brain Res 399:69-79

Mansour A., Khachaturian H., Lewis M.E., Akil H., and Watson S.J. (1988) Anatomy of CNS opioid receptors. TINS 11:308-314

Mansour A., Fox C.A., Burke S., Meng F., Thompson R.C., Akil H., and Eatson S.J. (1994a) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an *in situ* hybridization study. J Com Neurol 350:412-438

Mansour A., Fox C.A., Thompson R.C., Akil H., Watson S.J. (1994b) mu-opioid receptor mRNA expression in the rat CNS: comparision to mu-receptor binding. Brain Res 643:245-265

Mansour A., Fox C.A., Meng F., Akil H., and Watson S.J. (1994c) $\kappa 1$ receptor mRNA distribution in the rat CNS: comparision to κ receptor binding and prodynorphin mRNA. Mol Cell Neurosci 5:124-144

Margules D. L., Goldman B., and Finck A. (1979) Hibernation: an opioid-dependent state? Brain Res Bull 4:721-724

McMurray C. T., Pollock K. M., and Douglass I. (1992) Cellular and molecular anaylsis of opioid peptide gene expression. Molecular Approcahes to Drug Abuse Research Volume II: Structure, Function, and Expression. NIDA Research Monograph Vol 126. Lee T.N.H. (ed), pp 113-131, NIDA publication, Rockville.

Merchenthaler I. (1991) Co-localization of enkephalin and TRH in perifornical neurons of the rat hypothalamus that project to the lateral septum. Brain Res 544:177-180

Milanes M.V., Gonzalvez M.L., Fuente T., and Vargas M.L. (1991) Pituitary-adrenocortical response to acute and chronic addministration of U-50,488H in the rat. Neuropeptides 20:95-102

Milner T.A. and Veznedaroglu E. (1993) Serotonin-containing terminals synapse on septohippocampal neurons in the rat. J Neurosci Res 36:260-271

Minami M., Onogi T., Toya T., Katao Y., Hosoi Y., Maekawa K., Kataumata S., Yabuuchi K., and Saoh M. (1994) Molecular cloning and *in situ* hybridization histochemistry for rat mu-opioid receptor. Neurosci Res 18:315-322

Mitsuma T., Hirooka Y., and Nogimori T. (1993) Effects of immunoneutralization of endogenous opioid peptides on the hypothamalic-pituitary-thyroid axis in rats. Hormone Res 39:77-80

Moor R.Y. and Halaria A.E. (1975) Hippocampal innervation by serotonin neurons of the midbrain raphe in the rat. J Comp Neurol 164:171-183

Morley J.E., Elson M.K., Levine A.S., Shafer R.B. (1982) The effects of stress on central nervous system concentrations of the opioid peptide, dynorphin. Peptides 3:901-906

Morris B.J. and Herz A. (1989) Simultaneous down-regulation of κ binding sites and up-regulation of μ binding sites following chronic agonist/anatgonist treatment. Progress in Opioid Research. Advances in the Biociences Vol.75, Cros J., Meunier J.C., and Hamon M. (eds), pp 691-694, Pergamon Press, New York.

Mueller G.P., Stanton T.L., and Beckman A.L. (1990) Hibernation differentially affects beta-endorphin (B-END) and cholecystokinin octapeptide (CCK-8) concentrations in the pituitary and cental nervous system of ground squirrels. FASEB J 4:A733

Murakami N., Morimoto A., and Watanabe T. (1984) Radioactive deoxyglucose uptake in the rat CNS during thermoregulatory responses. Thermal Physiology Hales J.R.S. (ed), pp 105-108, Raven Press, New York.

Nabeshima T., Katoh A., Wada M., and Kameyama T. (1992) Stress-induced changes in brain met-enkephalin, leu-enkephalin and dynorphin concentrations. Life Sci 51:211-217

Naranjo J.R., Mellstrom B., Achaval M., and Sassone-Corsi P. (1991) Molecular pathways of pain: Fos/Jun-mediated activation of a noncanonical AP-1 site in the prodynorphin gene. Neuron 6:607-617

Negri M., Tonnarini G., Mariani R., Martino R., and Locuratolo N. (1989) Plasma met-enkephalin in physiopathological conditions. Opioid Peptides in Biological Fluids Genazzani A.R. and Negri M. (eds), pp 185-196, Parthenon, Lancs.

Nikolarakis K.E., Almeida O.F.X., Yassouridis A., and Herz A. (1989) Presynaptic auto- and allelo-receptor regulation of hypothalamic opioid peptide release. Neuroscience 31:269-273

Nizielski S.E., Levine A.S., Morley J.E., Hall K.A., and Gosnell B.A. (1986) Seasonal variation in opioid modulation of feeding in the 13-lined ground squirrel. Physiol Behav 37:5-9

North R.A., Williams J.T., Surprenant A., and Christie M.J. (1987) *mu* and *delta* receptors belong to a family of receptors that are coupled to potassium channels. Proc Natl Acad Sci USA 84:5487-5491

Novotona R., Jansky L., and Drahota Z. (1975) Effect of hibernation on turnover of serotonin in the brain stem of golden hamster (*Mesicricetus auratus*). Gen Pharmacol 6:23-26

Nurnberger F, Lee T. F., Jourdan M.L., and Wang L. C. H. (1991) Seasonal changes in methionine-enkephalin immunoreactivity in the brain of hibernator, *Spermophilus columbianus*. Brain Res 547:115-121

Olson G.A., Olson R.D., and Kastin A.J. (1994) Endogenous opiates: 1993. Peptides 15:1513-1556

Parent A., Descarries L., and Beaudet A. (1981) Organization of ascending serotonin systems in the adult rat brain. A radioautographic study after intraventricular administration of [^{3}H]5-hydroxytryptamine. Neuroscience 6:115-138

Passarelli T. and Costa T. (1988) *Mu* and *delta* opioid receptors inhibit serotonin release in rat hippocampus. J Pharmacol Exp Ther 248:299-305

Pelletier G.(1993) Regulation of proopiomelanocortin gene expression in rat brain and pituitary as studied by *in situ* hybridization. Annals New York Acad Sci 680:246-259

Polastron J., Meunier J.C., and Jauzac P. (1994) Chronic morphine induces tolerance and desensitization of mu-opioid receptor but not down-regulation in rabbit. Eur J Pharmacol 266:139-146

Popova N.K., Voronova I.P., and Kulikov A.V. (1993) Involvement of brian tryptophan hydroxylase in the mechanism of hibernation. Pharmacol Biochem Behav 46:9-13

Prather P.L., Tsai A.W., and Law P.Y. (1994) Mu and delta opioid receptor desensitization in undifferentiated human neurblastoma SHSY5Y cells. J Pharmacol Exp Ther 270:177-184

Rauru J., Pesonen U., Isaksson K., Huupponen R., and Koulu M. (1993) Effect of chronic treatment of TFMPP, a 5-HT$_1$ receptor agonist, on food intake, weight gain, plasma insulin and neuropeptide mRNA expression in obese Zuker rats. Eur J Pharmacol 234:191-198

Raynor K., Kong H., Hines J., Kong G., Benovic J., Yasuda K., Bell G.I., and Reisine T. (1994) Molecular mechanisms of agonist-induced desensitization of the cloned mouse kappa opioid receptor. J Pharmacol Exp Ther 270:1381-1386

Rector D.M., Poe G.R., and harper R.M. (1993) Imaging of hippocampal and neocortical neural activity following intravenous cocine adminstration in freely behaving rats. Neuroscience 54:663-641

Reiner A., Brauth S.E., Kitt C.A., and Quirion R. (1989) Distribution of mu, delta, and kappa opiate receptor types in the forebrain and midbrain of pigeons. J Comp Neurol 280:359-382

Remier S. and Hollt V. (1991) GABAergic regulation of striatal opioid gene expression. Mol Brain Res 10:49-54

Romsos D.R., Gosnell B.A., Morley J.E., and Levine A.S. (1987) Effects of kappa opiate agonists, cholecystokinin and nbombesin on intake of diets varying in carbohydrate- to -fat ratio in rats. J Nutrition 117:976-985

Romualdi P., Lesa G., and Ferri S. (1991) Chronic opiate agonists down-regulate prodynorphin gene expression in rat brain. Brain Res 563:132-136

Schick R. and Schusdziarra V. (1985) Physiological, pathophysiological and pharmacological aspects of exogenous and endogenous opiates. Clin Physiol Biochem 3:43-60

Sharif N.A. and Hughes J. (1989) Discrete mapping of brain mu and delta opioid receptors using selective peptides: quantitative autoradiography, speciese differences and comparison with kappa receptors. Peptides 10:499-522

Simantov R., Klein C., and Levy R. (1989) On mechanisms that control the expression of opioid receptors: effect of chronic neuronal activation and opioid antagonists. Progress in Opioid Research. Advances in the Biociences Vol.75, Cros J., Meunier J.C., and Hamon M. (eds), pp 329-332, Pergamon Press, New York.

Simon E.J. (1991) Opioid receptors and endogenous opioid peptides. Med Res Rev 11:357-374

Simpson J. A. and Fitch W. (1988) Applied Neurophysiology Wright, London.

Snyder S.H. (1986) Neuronal receptors. Ann Rev Physiol 48:461-471

Somjen G. (1983) Neurophysiology -the essentials p 122, Williams & Wilkins, Baltimore.

Spafford D.C. and Pengelley E.T. (1971) The influence of the neurohumoral serotonin on hibernation in the golden-mantled ground squirrel, *Citellus lateralis*. Comp Biochem Physiol 38A:239-250

Spencer R.L., Hruby V.J, and Burks T.F. (1990) Alteration of thermoregulatory set point with opioid agonists. J Pharmacol Exp Ther 252:696-705

Stanley B.G., Lanthier D., and Leibowitz S.H. (1989) Multiple brain sites sensitive to feeding stimulation by opioid agonists: a cannula-mapping study. Pharmacol Biochem Behav 31:825-832

Stanton T.L., Winokur A., and Beckman A.L. (1980) Reversal of natural CNS depression by TRH action in the hippocampus. Brain Res 181:470-475

Stanton T.L., Beckman A.L., Winokur A. (1981) Thyrotropin-releasing effects in the central nervous system: dependence on arousal state. Science 214:678-681

Stanton T.L., Winokur A., and Beckman A.L. (1982) Seasonal variation in thyrotropin-releasing hormone (TRH) content of different brain regions and the pineal in the mammalian hibernator, *Citellus lateralis*. Regul Pept 3:135-144

Stanton T.L., Craft C.M., and Reiter R.J. (1984) Decreases in pineal melatonin content during the hibernation bout in the golden-mantled ground squirrel, *Spermophilus lateralis*. Life Sci 35:1461-1467

Stanton T.L., Daley J.C.3d, and Salzman S.K. (1987) Prolongation of hibernation bout duration by continuous intracerebroventricular infusion of melatonin in hibernating ground squirrels. Brain Res 413:350-355

Stanton T.L., Caine S.B., and Winokur A. (1992) Seasonal and state-dependent changes in brain TRH receptors in hibernating ground squirrels. Brain Res Bull 28:877-886

Stengaard-Pedersen K. (1989) Opioid Peptides and Receptors Progress in Histochemistry and Cytochemicstry. Vol.20, No. 3, G Fischer, Stuttgart.

Strumwasser F. (1959) Regulatory mechanisms, brain activity and behaviour during deep hibernation in the squirrel, *Citellus beecheyi*. Am J Physiol 196:23-30

Tang F. and Mah W.S. (1991) The regional distribution of thyrotropin releasing hormone. leu-enkephalin, met-enkephalin, substance P, somatostatin and cholecystokinin in teh rat brain and pituitary. Neuropetidies 19:287-292

Ueda H., Fukushima N., Ge M., Takagi H., and Satoh M. (1987) Presynaptic opioid κ-receptor and regulation of the release of met-enkephalin in the rat brainstem. Neurosci Lett 81:309-313

Uhl G.R., Ryan J.P., and Schwartz J.P. (1988) Morphine alters preproenkephalin gene expression. Brain Res 459:391-397

Van Loon G.R., Kiritsy-Roy J., and Marson L. (1989) μ-opioid receptors in brain regulate basal and stress-induced sympathetic and parasympathetic neuronal activity and adrenomedullary secretion. Progress in Opioid Research. Advances in the Biociences Vol.75, Cros J., Meunier J.C., Hamon M. (eds), pp 611-614, Pergamon Press, New York.

Walker J.M., Thompson L.A., Frascella J., and Friederich M.W. (1987) Opposite effects of mu and kappa opiates on the firing-rate of dopamine cells in the substantia nigra of the rat. Eur J Pharmacol 134:53-59

Wang L.C.H. (1989) Ecological, physiological, and biochemical aspects of torpor in mammals and birds. Advances in comparative and environmental physiology Vol.4, Wang L.C.H. (ed), pp 361-401, Springer-Verlag, Berlin.

Wang J.Q. and Ingenito A.J. (1992) Centrally mediated cardiovascular actions of dynorphin A(1-8) on rat hippocampal formation. J Pharmacol Exp Ther 261:678-685

Wang J.Q. and Ingenito A.J. (1994) Cardiovascular effects of microinjection of dynrophin-A(1-8) into the hippocampus in conscious, spontaneously hypertensive and normotensive Wistar-Kyoto rats. Clin Exp Hypertens 16:229-243

Wiedel J.E. (1983) The formyl peptide chemotactic receptor: cellular processing of peptide and receptor. Receptor-Mediated Endocytosis. Receptors and Recognition Vol.15, Cuatrecasas P. and Roth T (eds), pp 275-299, Chapman and Hall, London.

Wilkinson M., Buchanan G.D., Jacobson W., Younglai E.V. (1986) Brain opioid receptors in the hibernating bat, *Myotis lucifugus*: modificantion by low temperature and comparsion with rat, mouse and hamster. Pharmacol Biochem Behav 25:527-532

Wollemann M. (1990) Endogenous Opioids and Cyclic AMP. Prog Neurobiol 16:145-154

Wollemann M., Benyhe S., and Simon J. (1993) The kappa-opioid receptor: evidence for the different subtypes. Life Sci 52:599-611

Wunnenberg W., Merker G, and Speulda E. (1978) Thermosensitivity of preoptic neurons and hypothalamiic integrative function in hibernators and nonhibernators. Strategies in cold: natural torpidity and thermogenesis Wang L.C.H. and Hudson J.W. (eds), pp 267-297, Academic Press, New York.

Yeadon M. and Kitchen I. (1989) Opioids and respiration. Prog Neurobiol 33:1-16

Yebenes E.G. and Pelletier G. (1993) Opioids regualtion of proopiomelanocortin (POMC) gene ecpression in the rat brain as studied by *in situ* hybridization. Neuropeptides 25:91-94

Yim G.K.W. and Lowy M.T. (1984) Opioids, feeding, and anorexias. Federation Proc 43:2893-2897

Yoshioka M., Matsumoto M., Togashi H., Smith C.B., and Saito H. (1993) Opioid receptor regulation of 5-hydroxytryptamine release from the rat hippocampus measured by in vivo microdialysis. Brain Res 613:74-79

Yu L.C. and Cai Y.P. (1993) Effects of opioid receptor antagonists administration to suprachiasmatic nucleus on hibernation of ground squirrels *Citellus Dauricus*. Comp Biochem Physiol 104C:249-252

Zeisberger E. (1990) The role of septal peptides in thermoregulation and fever. Thermoreception and Temperature Regulation Bligh J. and Voigt K. (eds), pp 273-283, Springer-Verlag, Berlin.

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PUBLICATIONS AND WORKS IN PROGRESS:

Cui, Y., Lee, T.F., and Wang, L.C.H. (1994) Species difference in the modulatory effect of κ agonist on 5-HT release from ground squirrel and rat hippocampus. Neurosci. Lett. 175:126-128.

Wang, L.C.H., Cui, Y., and Lee T.F. (1994) Is the hypothalamic serotonergic system involved in septal 5-HT-induced hypothermia in rats ? In: *Thermal Balance in Health and Disease: Recent Basic Research and Clinical Progress (Advances in Pharmacological sciences).* Birkhäuser Verlag Basel, 469-473.

Cui, Y., Lee, T.F., Kramarova, L.I., and Wang, L.C.H. (1993) The modulatory effects of *mu* and *kappa* opioid agonists on 5-HT release from hippocampal and hypothalamic slices of euthermic and hibernating ground squirrels.

Life Sci. 53:1957-1965.

Cui, Y., Lee, T.F., and Wang, L.C.H. (1993) Thermoregulatory responses following injection of 5-hydroxytryptamine into the septohippocampal complex in rats. Pharmacol. Biochem. and Behav. 45:935-939.

Kramarova, L.I., Lee, T.F., Cui, Y., and Wang, L.C.H. (1991) State-dependent variation in the inhibitory effect of [D-Ala^2,D-Leu^5]-enkephalin on hippocampal serotonin release in ground squirrels. Life Sci. 48:175-181.

Cui, Y., Lee, T.F., Kramarova, L.I., and Wang, L.C.H. (1993) The effect of *mu, delta, and kappa* opioid agonists on 5-HT release from hippocampal slices of euthermic and hibernating ground squirrels. In: *Living in the cold III: international hibernation symposium at Mt. Crested Butte, Colorado, U.S.A., October 2-8, 1993,* p12.

Cui, Y., Lee, T.F., and Wang, L.C.H. *In vivo* microdialysis study on changes in septal dynorphin and β-endorphin activities in active and hibernating Columbian ground squirrels. (manuscript submitted to Brain Res.)

Cui, Y., Lee, T.F., and Wang, L.C.H. State-dependent changes of brain endogenous opioids in mammalian hibernation. (manuscript submitted to Brain Res. Bull.)

Cui, Y., Lee, T.F., Westly, J., and Wang, L.C.H. State-dependent changes in *mu, delta,* and *kappa* receptor binding in the limbic system of Columbian ground squirrel in hibernation. (manuscript in preparation)

Cui, Y., Lee, T.F., and Wang, L.C.H. State-dependent change in the thermoregulatory response to intrahippocampal injection of exogenous δ and κ agonists to Columbian ground squirrels. (manuscript in preparation)

Cui, Y., Lee, T.F., and Wang, L.C.H. *In vivo* microdialysis investigation of state-dependent change in the stimulatory effect of exogenous δ on 5-HT apparent turnover in the septum of Columbian ground squirrels. (manuscript in preparation)

PRESENTATIONS:

"Changes in endogenous opioid activity in mammalian hibernation", University of Alberta, April 1995

"The effect of *mu, delta, and kappa* opioid agonists on 5-HT release from hippocampal slices of euthermic and hibernating ground squirrels", Mt. Crested Butte, Colorado, October 1993

"State-dependent changes in the modulatory role of opioids on K^+- evoked 5-HT release from the hippocampus of Richardsons' ground squirrels", University of Alberta, February 1993